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RESEARCH ARTICLE

Protein profiling of genomic instability in endometrial cancer

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Abstract DNA aneuploidy has been identified as a prognostic factor in the majority of epithelial malignancies. We aimed at identifying ploidy-associated protein expression in endometrial cancer of different prognostic subgroups. Comparison of gel electrophoresis-based protein expression patterns between normal endometrium ($n = 5$), diploid ($n = 7$), and aneuploid ($n = 7$) endometrial carcinoma detected 121 ploidy-associated protein forms, 42 differentially expressed between normal endometrium and diploid endometrioid carcinomas, 37 between diploid and aneuploid endometrioid carcinomas, and 41 between diploid endometrioid and aneuploid uterine papillary serous cancer. Proteins were identified by mass spectrometry and evaluated by Ingenuity Pathway Analysis. Targets were confirmed by liquid chromatography/mass spectrometry. Mass spectrometry identified 41 distinct polypeptides and pathway analysis resulted in high-ranked networks with vimentin and Nf-κB as central nodes. These results identify ploidy-associated protein expression differences that overrule histopathology-associated expression differences and emphasize particular protein networks in genomic stability of endometrial cancer.

Keywords Aneuploidy · Endometrial carcinoma · Genomic instability · Mass spectrometry · Pathway analysis · Two-dimensional gel electrophoresis

Abbreviations

2-DE	Two-dimensional gel electrophoresis
Ae	Aneuploid endometrioid
Ambic	Ammoniumbicarbonate
Au	Aneuploid uterine papillary serous cancer
CGH	Comparative genomic hybridization
De	Diploid endometrioid
EnCa	Endometrial cancer
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
IPA	Ingenuity Pathway Analysis
LC–MS/MS	Liquid chromatography and tandem mass spectrometry
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry

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PCA	Principal component analysis
UPSC	Uterine papillary serous cancer

Introduction

Endometrial cancer (EnCa) is a common malignancy and accounts for about 6% of all female malignancies in the Western world. Due to early bleeding symptoms, about 70% of affected women are detected at tumor stage I when the tumor is limited to the corpus uteri and mean survival is close to 87%. Next to histopathology, tumor stage, tumor grade, and DNA ploidy are used as prognostic factors with aneuploidy being significantly related to an increased risk of nodal metastases, recurrence, and disease-related death [1, 2]. For example, uterine papillary serous carcinoma (UPSC) has an inferior prognosis compared to that of endometrioid carcinomas [3]. The prognostic potential of DNA ploidy was shown in a study involving 376 endometrial carcinoma patients with a more favorable 5-year survival rate of 94% for patients with diploid cell populations as opposed to those with aneuploid malignancies (83%) [4].

DNA ploidy can be assessed by flow or image cytometry [5], the latter case assessing cells on tissue sections [6]. We demonstrated for breast carcinomas that not only the ploidy status per se but also the grade of genomic instability has an impact on patient prognosis [7]. Instability can be assessed by detection of gains and losses of chromosomal regions. For instance, in breast carcinomas, a gain of 1q is a common alteration, whereas for vaginal carcinomas, the most frequent aberration is a gain of 3q [8, 9]. In endometrial carcinomas, significant allelic gains have been reported for many chromosome arms, such as 1q, 3q, 8q, and 10q [10–12]. The predominance of these tumor-specific chromosomal aneuploidies is associated with increased expression of genes located on particular chromosomes in a manner that seems to be independent of tissue and cell type [13]. The multitude of chromosomal aberrations seems to lead to an irreversible disturbance of transcriptional regulation and a loss of genomic integrity in aneuploid tumor cells [14]. Furthermore, we found that aneuploidy-associated genomic instability also translates into protein expression alterations in colorectal cancer cell lines [15]. However, acquiring knowledge of the underlying molecular network mechanisms characterized by chromosomal abnormalities remains a major challenge in the EnCa to elucidate.

In the present study, we identified genomic instability-associated protein expression patterns of interest for risk evaluation and therapy in endometrial carcinomas.

Materials and methods

Patients, sample collection, and preparation

Endometrial tumor material and normal endometrium were collected after hysterectomies for EnCa or benign affections. Collection was performed between 1997 and 2003 after patient consent and according to ethical review board approvals (#00-385 and #03-034) at the Karolinska Hospital, Stockholm, Sweden. Clinical data of 14 malignancies and five normal samples are provided in S1 and S2. Cells were immediately after collection harvested from the surface of the tumor and the normal epithelium by scalpel scraping [16]. This technique supplies a high percentage of representative cells for analysis. After preparation, each sample was quality checked by comparison of Giemsa-stained smears with histological slides. Only samples with more than 95% tumor cells were used. The samples were stored at −80°C until two-dimensional gel electrophoresis (2-DE).

Histopathology and image cytometry

Histopathological subtype and degree of differentiation were evaluated according to the FIGO grading system [17]. Ten tumors were of endometrioid subtype and four of the less common UPSC type. DNA ploidy determination was performed on paraffin-embedded tumor specimens of Feulgen-stained histopathological sections (8 µm) using image cytometry as described [5]. At least 100 nuclei per sample were selected interactively and their DNA contents measured using the ACAS image system (Ahrens ACAS, Hamburg, Germany). In addition, small lymphocytes were measured as internal controls. The classification of the DNA profiles was performed according to Auer et al. [5].

Two-dimensional gel electrophoresis

Protein concentrations of samples were determined in quadruplicates by addition of 25 µl of concentrated assay reagent (Bio-Rad) to 1 µl solubilized sample diluted in 100 µl Milli-Q water using 96-well microplates [18] followed by isoelectric focusing and SDS-PAGE of 75-µg samples as described [16] (S3). Gels were stained with silver nitrate, scanned with a flatbed densitometer (GS 710, Bio-Rad), and analyzed with PDQuest software (Bio-Rad, Hercules, CA, USA, version 8.0.1).

Statistical analysis

Only protein spots that showed expression in at least 50% of the samples within each ploidy group were used for statistical analyses by Welch *t* tests for unequal variances

to compare protein expression in four analytical sets, i.e., those of (1) normal endometrium versus diploid endometrioid cancer, (2) diploid versus aneuploid endometrioid cancer, (3) aneuploid endometrioid cancer versus aneuploid UPSC, and (4) diploid endometrioid cancer versus aneuploid UPSC [19]. For the pairwise comparison, the significance level was set to 0.001, whereas 0.01 was used for the remaining tests due to low sample size. To detect proteins with increasing or decreasing expression from normal endometrial versus the tumor tissues, an exact version of the Jonckheere–Terpstra test was applied at a significance level of 0.001. Fold change was calculated as logarithmized means of each group. Principal component analysis (PCA) was used to discriminate normal mucosa and carcinomas with the software package R version 2.10.1 for statistical analyses with R packages clinfun version 0.8.10 (Jonckheere–Terpstra test) and FactoMineR version 1.14 (PCA).

In-gel digestion and mass spectrometry (MALDI-TOF)

2-DE gel spots were manually cut with a scalpel in a laminar air-flow bench. After destaining and digestion with trypsin as described [20] (S4), the fragments were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) in an Ultraflex III TOF/TOF instrument (Bruker Daltonics, Bremen, Germany).

Network and functional pathway analysis

Differentially expressed and identified proteins were analyzed by the Ingenuity Pathway Analysis software (Ingenuity, Mountain View, CA, USA; http:\\www.ingenuity.com, version 8.5-2803). The IPA-generated networks are ranked using a score that is calculated as the negative logarithm of the p value. This p value indicates the likelihood of the proteins of interest being found together within a network. ANXA4, ANXA5, HSP90AB1, KRT9, KRT10, and PDIA3 were identified from at least two protein spots and showed non-homogenous up- or down-regulation between groups because of possible post-translational modification and were excluded from pathway analysis. For the evaluation of aneuploid endometrioid cancer versus aneuploid UPSC, only one differentially expressed protein was detected and no relevant network analysis was performed.

Confirmation by liquid chromatography and tandem mass spectrometry (LC–MS/MS)

Picked protein spots were processed and digested with trypsin using a robotic protein handling system (MassPREP, Waters). Peptides were extracted with 30 µl of 5% formic acid/2% acetonitrile and subsequently with 24 µl 2.5% formic acid/50% acetonitrile. Tryptic fragments were analyzed using a quadrupole time-of-flight mass spectrometer (Q-TOF Premier API, Waters, Milford, MA, USA) with a standard Z-spray source coupled to a Waters nanoAcquity system (S5).

Results

Genomic instability

DNA image cytometry showed seven of the 14 carcinomas to be of diploid endometrioid histopathology and the remaining seven to be aneuploid with endometrioid ($n = 3$) and UPSC ($n = 4$) histopathology. Based on clinical routine assessment of more than 25 normal endometrial samples, diploidy was presumed for the normal endometrium being part of this study.

Two-dimensional gel electrophoresis and pathway analysis

PCA results showed an overall clustering of each of the normal endometrial mucosa ($n = 5$), diploid endometrioid cancer ($n = 7$), and aneuploid EnCa samples, including the endometrioid ($n = 3$) and UPSC ($n = 4$) subtypes. However, two samples, CP 27 (aneuploid UPSC) and CP 35 (diploid endometrioid cancer), showed aberrant locations in the PCA plot and were therefore excluded from analysis, resulting in a perfect separation of all three groups (S2, S6).

Normal endometrium versus diploid endometrioid carcinomas

A total of 42 differentially expressed proteins were detected ($p \leq 0.001$); 19 (45%) were identified by MALDI-TOF-MS: seven over- and 12 under-expressed in the diploid group (Fig. 1, S7). These up- and down-regulated proteins were further analyzed using the Ingenuity Pathway Knowledge Base (IPA) generating networks of interaction. The first network with a score of 33 is associated with Cellular Assembly and Organization and Small Molecule Biochemistry, the second with a score of 10 with Development and Function, Cell Morphology, Skeletal and Muscular System Development and Function. Vimentin (VIM), β-actin (ACTB), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) are central nodes of these networks. These three proteins are associated with diseases and functions related to cancer, neurological disease, and genetic disorders ($p < 0.00001$ to $p < 0.0464$; Fig. 2a, S8). Interestingly, both networks are connected via RAPGEF3 (Fig. 2a).

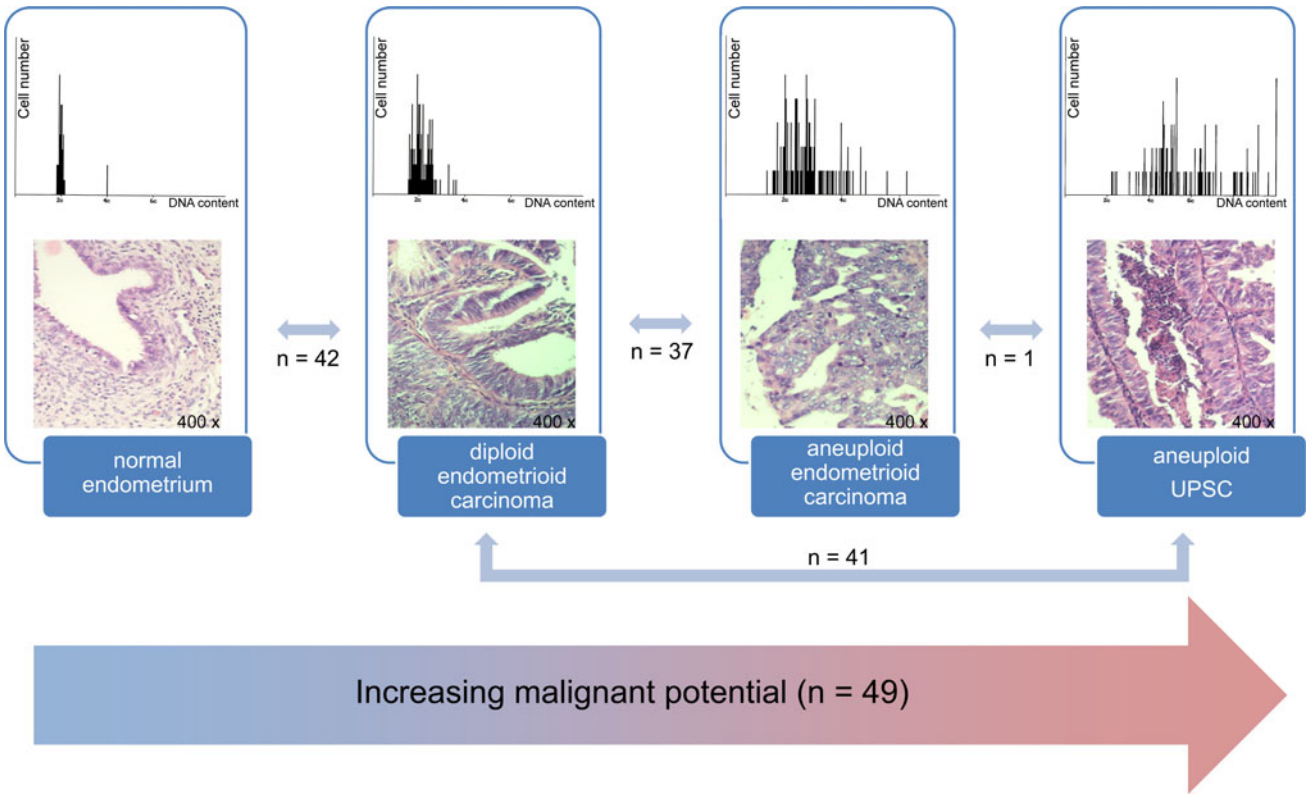

Fig. 1 Histopathological subtypes and ploidy groups. *Numbers* show differentially expressed protein spots in the statistical pairwise (*top*) and trend (*bottom*) tests. DNA histograms show DNA content on the *x*-axis and the total number of cell on the *y*-axis

Diploid versus aneuploid endometrioid carcinomas

A total of 37 differentially expressed protein spots were detected, 20 (54%) identified by MALDI-TOF: 17 higher- and three lower-expressed in the aneuploid group (Fig. 1, S9), and ten eligible for IPA analysis interacting in a network (score 25) associated with Cellular Assembly and Organization, Nucleic Acid Metabolism, and Small Molecule Biochemistry. Vimentin, growth factor receptor-bound protein 2 (GRB2) and β-actin are central nodes of this network and associated with diseases and functions regarding cancer, neurological disease, and genetic disorders ($p < 0.00001$ to $p < 0.0486$) and cellular growth and proliferation and cellular assembly and organization ($p < 0.00001$ to $p < 0.0382$; Fig. 2b, S8).

Diploid endometrioid cancer versus aneuploid UPSC

A total of 41 spots were differentially expressed ($p \leq 0.01$), 15 (37%) identified by peptide mass fingerprinting: ten higher- and five lower-expressed in the aneuploid group (Fig. 1, S10), and involved in a network associated with Lipid Metabolism, Small Molecule Biochemistry, and Cell Morphology (score 25). The differentially expressed β-actin, annexin A2 (ANXA2), and the heterogeneous nuclear ribonucleoprotein K isoform A (HNRNPK) are identified as major central nodes of this network and associated with cellular functions of cancer, gastrointestinal disease, and inflammatory response ($p < 0.00001$ to $p < 0.0479$; Fig. 2c, S8).

A network comparison between all significant networks mentioned above showed eight proteins in common (β-actin, ATP synthase 5B (ATP5B), ATP5E, insulin, IVNS1ABP, lamin b, PLS1, and vimentin) that connect the network of the normal endometrium versus diploid endometrioid carcinomas with the network of the diploid versus aneuploid endometrioid carcinomas. A total of 16 proteins (β-actin, γ-actin (ACTG1), actin, annexin A2, CAP2, EPS8L1, EPS8L2, GAS8, HIP1R, NCALD, PHACTR1, PLS1, PRS13, PRS18, SSH1, VIL1) associate the diploid versus aneuploid endometrioid carcinomas network with the diploid endometrioid cancer versus aneuploid UPSC network.

Trend analysis of normal endometrium versus diploid endometrioid carcinomas, aneuploid endometrioid carcinomas and aneuploid UPSC

A total of 49 proteins were detected with an increase or decrease over all four groups ($p \leq 0.001$), 31 spots (63%)

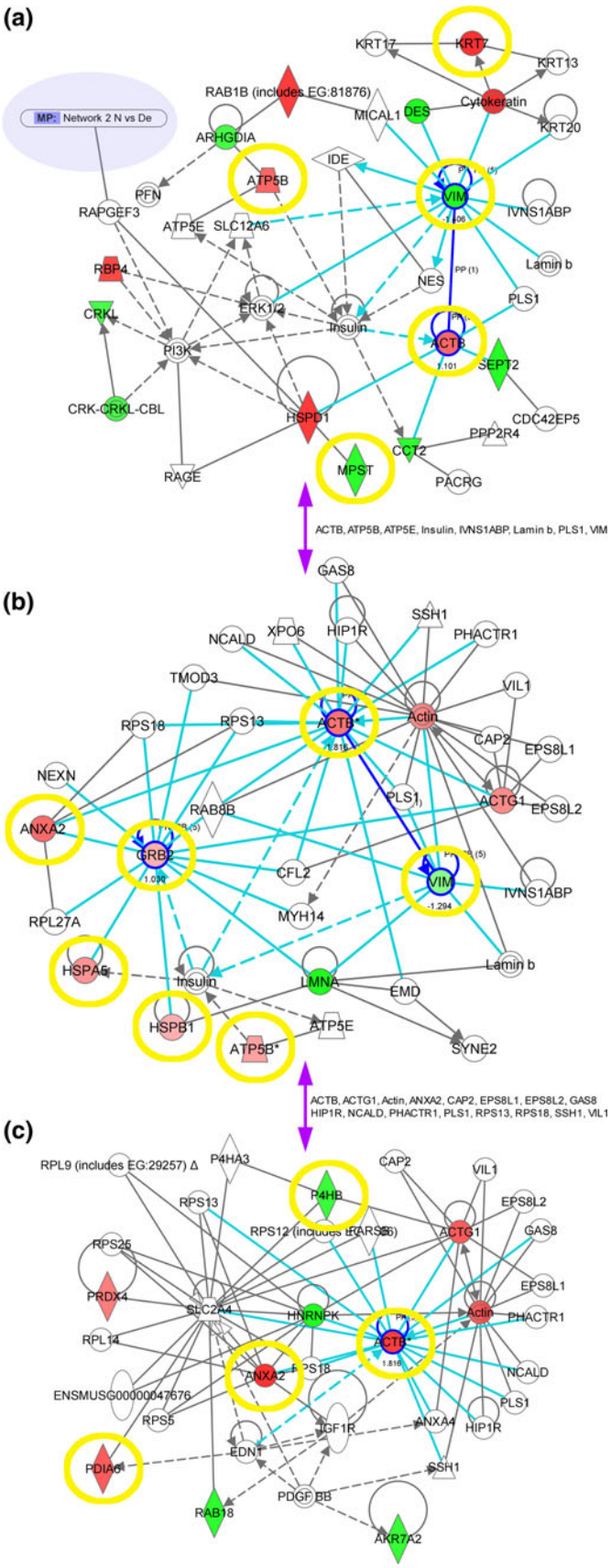

Fig. 2 Networks of differentially expressed proteins. Normal mucosa and diploid endometrioid carcinoma are compared in **a**, diploid endometrioid and aneuploid endometrioid carcinoma in **b**, and diploid endometrioid carcinoma and aneuploid UPSC in **c**. *Red* and *green designations* indicate over- and under-expressed proteins, *blue arrows* and *circulations* central nodes of the networks, *encircling in yellow* LC–MS/MS-verified evaluations, and *arrows in purple* those proteins that connect different networks

identified, ten down-regulated and 21 up-regulated towards the aneuploid groups (Fig. 1; Table 1). Two overlapping networks interact with Cellular Function and Maintenance, Cellular Compromise and Nucleic Acid Metabolism (score 29); and Lipid Metabolism, Molecular Transport and Small Molecule Biochemistry (score 18). NF-κB, mitogen-activated protein kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (P38MAPK) act as central nodes and were found to be associated with cellular functions regarding cancer, gastrointestinal disease, inflammatory response as well as with cellular function and maintenance ($p < 0.00001$ to $p < 0.0476$; Fig. 3, S8).

Confirmation by liquid chromatography/tandem mass spectrometry (LC–MS/MS)

Based on fold changes, pathway analysis and molecular functions, 28 protein identities were selected for confirmation by LC–MS/MS, 14 with a higher and 14 with a lower expression in the aneuploid carcinomas relative to the diploid carcinomas. Nineteen protein identities were then confirmed by LC–MS/MS, while spectra of nine proteins (RAB1B, ARHGDIA, RBP4, CRKL, MPST, ACTG1, PRDX4, HNRNPK, RAB18) showed no identification.

In summary, we could confirm our initial protein identifications by LC-MS/MS for 19 of 28 (68%) proteins. Repeated IPA pathway analysis after exclusion of the non-validated nine protein identities did not change results regarding molecular functions in different ploidy and histology subtypes of tumors.

Discussion

In the present study, we focused on differences between protein profiles in diploid and aneuploid EnCa and normal endometrium. The results provide molecular relationships with network differences in relation to polyploidization.

Evaluation with a 2-DE approach showed 121 distinct protein spots with significant expression differences within all defined subgroups. We observed that aneuploid EnCas are distinct in their 2-DE protein expression profiles from diploid tumors and normal endometrium. We identified 69 polypeptides (57%), representing 41 distinct proteins, the majority connected in networks as determined by pathway analysis. The functions extracted from these networks concerned cellular function and maintenance, cancer, gastrointestinal disease, cell morphology, and small molecule biochemistry, with NF-κB, vimentin, and β-actin as central nodes. The identification of differentially expressed proteins in top pathways in connection of networks with cell functions strongly suggests that these proteins should be submitted to extensive downstream assessment.

Interestingly, we detected only one protein spot that distinguishes aneuploid endometrioid cancer from aneuploid UPSC. Along with the fact of more significant spots between samples of the same histopathology but different ploidy status, we suggest that aneuploidy could be of greater impact regarding poor prognosis than the histological subtype. Interpretations from other data also highlight the importance of aneuploidy for the prognosis [21].

All 69 proteins identified with altered expression changes belong to several functional groups. For instance, the LC–MS/MS-identified β-actin, γ-actin, annexin A2, and ATP synthase 5B concern functions of cellular growth and proliferation and thus reflect different rates of cell division between diploid and aneuploid carcinomas. Another group includes proteins involved in cell-to-cell signaling and interaction, e.g., the serine peptidase inhibitors. A third group, with increased expression levels in the diploid and aneuploid samples was identified as a member of the heat shock protein (HSP) family. HSPs are abundant intracellular polypeptides up-regulated by stress (e.g., temperature, cancer, infection). Their release is considered as a putative danger signal and maintains the structural and metabolic integrity of the cells. Several reports using therapeutic cancer vaccines based on HSPs have been published [22, 23] showing HSP27 overexpression to be a good independent prognostic indicator ($p < 0.005$) in patients with endometrial adenocarcinoma [24]. Up-regulation of HSP27 has also been described as a potential marker for primary vaginal carcinoma [25] and as immunohistochemical tumor marker in the endometrium [26]. This member of the heat shock family is up-regulated in our comparison between diploid and aneuploid endometrioid tumor samples and was confirmed by LC–MS/MS. It is considered as a putative biomarker for polyploidization, regardless of the histopathological background, and reflects structural and metabolic disintegrity of cells. In addition, up-regulation of HSP27 is associated with aggressive tumor behavior, as shown by the fact that only poorly differentiated tumors overexpress HSP27, and that the level of up-regulation in Barrett's esophagus is higher in samples presenting lymph node metastasis [27]. This suggests that the higher the level of expression, the more aggressive the tumor. This conclusion is in agreement with results from analyses of also gastric and esophageal squamous cell carcinomas [28, 29] and with our present finding of aneuploid EnCas with increased HSP27 expression.

Yet, another group consists of proteins involved in mechanisms of genetic disorders and maintenance of genomic stability. We found tyrosine 3-monooxygenase/tryptophan-5-monooxygenase activation protein, epsilon

Table 1 Identifications of the trend analysis

Protein name	Accession number	Sequence coverage (%)	Matched/ searched peptides	Score	Trend (N-De-Ae-Au)	Fold change (logarithmized)
Lower expression in aneuploid UPSC						
Keratin 10[a]	AAH34697	15	6/47	0.81	↓	0.73
14-3-3 ε	NP_006752	35	11/61	0.011	↓	0.82
Cytokeratin 9[a]	CAA82315	18	6/73	0.83	↓	0.51
Vimentin	AAA61279	68	39/96	2.8×10^{-5}	↓	0.85
Annexin A5[a]	NP_001145	56	19/96	5.3×10^{-4}	↓	0.84
Thioredoxin-like 1	NP_004777	43	8/93	0.034	↓	0.82
Vimentin	NP_003371	45	22/68	1.6×10^{-4}	↓	0.64
HSP90 alpha (cytosolic), class B[a]	NP_031381	28	17/57	2.7×10^{-4}	↓	0.35
Ribosomal protein P0	NP_000993	48	10/34	1.7×10^{-5}	↓	0.82
Annexin A4[a]	NP_001144	19	5/34	0.41	↓	0.77
Higher expression in aneuploid UPSC						
Annexin A5[a]	NP_001145	26	8/33	0.015	↑	1.34
ATP-Synthase 5B	NP_001677	15	5/43	0.33	↑	1.82
HSP70	CAI18465	10	3/40	0.73	↑	1.66
β-actin	AAH12854	28	7/33	1.8×10^{-3}	↑	1.61
β-actin	AAH12854	28	7/33	3.7×10^{-4}	↑	1.56
β-actin	AAH12854	28	7/33	9.4×10^{-4}	↑	1.85
HSP90 alpha (cytosolic), class B[a]	NP_031381	28	17/57	0.16	↑	1.44
HSP60	AAA36022	21	8/75	0.28	↑	1.64
Cytokeratin 9[a]	CAA82315	18	6/73	1.3×10^{-3}	↑	1.87
Keratin 10[a]	AAH34697	15	6/47	0.018	↑	1.51
ATP-synthase 5B	NP_001677	15	5/43	0.047	↑	1.45
γ-actin	AAH10417	31	4/46	0.21	↑	1.51
Prot disulfide-isomerase A6	NP_005733	31	9/52	7.3×10^{-4}	↑	1.58
HSP60	AAA36022	21	8/75	0.65	↑	1.49
HSP70 protein 5	NP_005338	26	14/100	2.8×10^{-5}	↑	1.90
β-actin	AAH12854	28	7/33	2.0×10^{-7}	↑	1.39
Annexin A2	AAH09564	43	15/53	2.9×10^{-4}	↑	1.94
Phospholipase C beta 4 isoform	NP_000924	10	4/39	0.64	↑	1.50
HSP27	NP_001531	25	5/43	0.74	↑	1.35
Protein disulfide-isomerase family A member 3[a]	NP_005304	23	10/48	3.4×10^{-4}	↑	1.71
Annexin A4[a]	NP_001144	52	21/64	5.9×10^{-6}	↑	1.33

[a] Six proteins (ANXA4, ANXA5, KRT9, KRT10, PDIA3 and HSP90AB1) were identified out of at least two protein spots and showed non-homogenous up- or down regulation between groups due to possible post-translational modification and were thus excluded from all pathway analysis

N normal, *De* diploid endometrioid, *Ae* aneuploid endometrioid, *Au* aneuploid UPSC

(14-3-3-ε) and vimentin to be less expressed in aneuploid samples. Both protein identifications were confirmed by LC–MS/MS. The 14-3-3-ε protein encoded by the YWHAE gene, binds to phosphoserine-containing proteins and mediates signal transduction. For example, 14-3-3 binding is required for the stabilization of active RAF-1 [30] and CDC25-mediated cell cycle control [31], whereas its interaction with BAD (BCL2-associated agonist of cell death) and BAX (BCL2-associated X protein) prevents their proapoptotic release to the mitochondrial membrane [32, 33]. Furthermore, we found NF-κB as a central node in our pathway analysis. This is consistent with other findings linking NF-κB with EnCa [34]. The NF-κB signal transduction pathway plays important roles in the establishment and maintenance of cell phenotypes, through regulation of the expression of many genes. NF-κB has also been shown to regulate the epithelial-mesenchymal transition marker vimentin, which is down-regulated in aneuploid samples

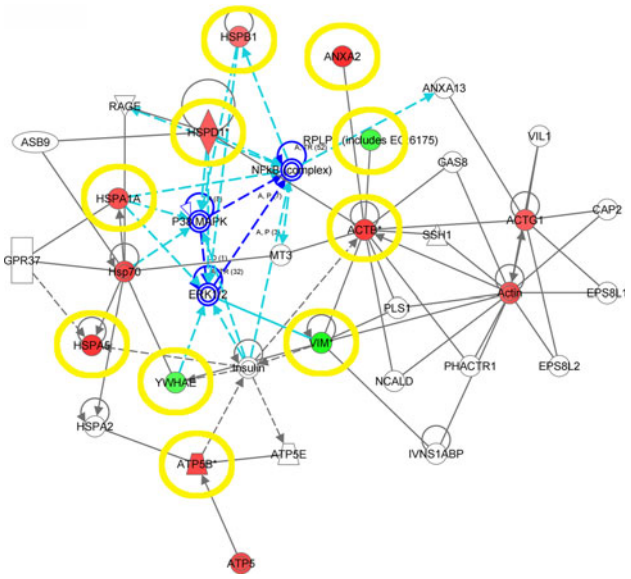

Fig. 3 Network for continuous trends in expression of differentially expressed proteins. Compared are proteins from normal endometrium over diploid endometrioid carcinomas and aneuploid endometrioid carcinomas to aneuploid UPSC. Proteins denoted in *red* are overexpressed and those in *green* underexpressed in the UPSC group. *Blue arrows* and *encirclings* indicate central nodes of the networks, *yellow ovals* LC–MS/MS-verified proteins

[35, 36] and required to maintain cellular integrity [37, 38]. Hence, our finding of higher expression of vimentin in diploid endometrioid carcinomas is in agreement with all data, and down-regulation of vimentin is concluded to be of importance for genomic instability and polyploidization.

Particular attention should be drawn to one protein, thioredoxin-like 1 (TXNL1), now verified by LC–MS/MS. This protein cannot be classified into the groups mentioned above. It has been reported that TXNL1 overexpression can increase the transcriptional repressor function through its binding to the transcription factor B-Myb [39] and that TXNL1 is down-regulated in aneuploid colorectal cancer cell lines and primary colorectal cancer [15]. We therefore now conclude that higher TXNL1 expression might be important to maintain genomic stability.

In conclusion, using a proteomic approach to identify protein signatures of genomic stability/instability irrespective of histological subgroups we have identified 41 distinct polypeptides. Although 2-DE represents a subset analysis of the overall proteome, we were able to deliver a complex map of intact proteins, in relation to molecular networks, that reflect aneuploidy-associated protein expression alterations available for clinical testing. The agreements with the data for other tumors suggest that the differentially expressed proteins follow mechanisms and pathways in common, independent of histopathology or tumor entity.

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Conflict of interest All authors declare that they have no competing financial interests.

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