Impact of genomic stability on protein expression in endometrioid endometrial cancer

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BACKGROUND: Genomic stability is one of the crucial prognostic factors for patients with endometrioid endometrial cancer (EEC). The impact of genomic stability on the tumour tissue proteome of EEC is not yet well established.

METHODS: Tissue lysates of EEC, squamous cervical cancer (SCC), normal endometrium and squamous cervical epithelium were subjected to two-dimensional (2D) gel electrophoresis and identification of proteins by MALDI TOF MS. Expression of selected proteins was analysed in independent samples by immunohistochemistry.

RESULTS: Diploid and aneuploid genomically unstable EEC displayed similar patterns of protein expression. This was in contrast to diploid stable EEC, which displayed a protein expression profile similar to normal endometrium. Approximately 10% of the differentially expressed proteins in EEC were specific for this type of cancer with differential expression of other proteins observed in other types of malignancy (e.g., SCC). Selected proteins differentially expressed in 2D gels of EEC were further analysed in an EEC precursor lesion, that is, atypical hyperplasia of endometrium, and showed increased expression of CLIC1, EIF4A1 and PRDX6 and decreased expression of ENO1, ANXA4, EMD and Ku70.

CONCLUSION: Protein expression in diploid and aneuploid genomically unstable EEC is different from the expression profile of proteins in diploid genomically stable EEC. We showed that changes in expression of proteins typical for EEC could already be detected in precursor lesions, that is, atypical hyperplasia of endometrium, highlighting their clinical potential for improving early diagnostics of EEC.

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Endometrial cancer (EC) is the fourth most common gynaecologic malignancy in Europe and Northern America. Even if detected at stage I, EC relapses in the majority of these cases (Creasman et al., 2006). Thus, diagnostics for detecting asymptomatic EC and precancer lesions is of paramount importance (Buchanan et al., 2009; Seebacher et al., 2009). EC is divided into oestrogen-dependent endometrioid EC (EEC) that develops from atypical hyperplasia of endometrium (AH) and oestrogen-independent nonendometrioid EC that is usually characterised by a poorer prognosis (Bokman, 1983; Horn et al., 2007). An important factor that defines the aggressiveness of malignancies, including EC, is chromosomal stability. More than half of the cases of EC are genomically stable and diploid (Lundgren et al., 2002, 2004). In comparison, all squamous cervical cancers (SCCs) and the vast majority of epithelial ovarian cancers are genomically unstable and aneuploid. Expression of proteins in diploid and aneuploid EC differs significantly (Lundgren et al., 2004). Characterisation of these proteins may provide new biomarkers for improved early diagnostics of EC and precursor lesions.

Proteomics is a potential method in the search for new cancer markers (Pitteri and Hanash, 2010; Sharon et al., 2010). Several proteomics-based studies of EC revealed important information about the endometrium, that is, the impact of genomic instability in endometrial cancer on protein expression (Lundgren et al., 2006), the proteome involved in myometrial invasion of endometrial cancer (Monge et al., 2009), and new insights into the secretome of endometrium (Casado-Vela et al., 2009). Unfortunately, only a few of the proteins identified in these studies were further analysed for their clinical value. Also, in many cases a comparison is only made between cancer and the respective normal tissue, without comparison with other closely related malignancies. Thus, the cancer specificity of the identified proteins could not be determined (Petrak et al., 2008). Furthermore, the similarities observed between protein expression in EEC and precursor lesions may be used for early detection of EEC.
Table 1 Description of clinical material used for (a) 2D gel electrophoresis and (b) immunohistochemical analysis

(a) Sample ID TNM Stage, FIGO, 1988 Ploidy Age

| No. | Sample ID | Tumor, Node, and Metastasis | Stage, FIGO, 1988 | Ploidy | Age |
|-----|-----------|-----------------------------|-------------------|--------|-----|
| 1   | Gs1       | T1aN0G1                     | IA                | D5     | 54  |
| 2   | Gs2       | T1aN0G2                     | IA                | D5     | 82  |
| 3   | Gs3       | T1aN0G2                     | IA                | AS     | 51  |
| 4   | Gs4       | T1bN0G1                     | IB                | D5     | 69  |
| 5   | Gs5       | T1bN0G1                     | IB                | D5     | 86  |
| 6   | Gs6       | T1bN0G1                     | IB                | AS     | 84  |
| 7   | Gs7       | T1bN0G3                     | IC                | D5     | 69  |

...Continued

(b) Sample ID TNM Stage, FIGO, 1988 Relapse, months Overall survival, months

| No. | Sample ID | Tumor, Node, and Metastasis | Stage, FIGO, 1988 | Relapse, months | Overall survival, months |
|-----|-----------|-----------------------------|-------------------|-----------------|--------------------------|
| 16  | CC1       | T1bN0G3                     | IC                | 22 (local)      | 24                       |
| 17  | CC2       | T1bN0G3                     | IB                | 10 (distant)    | 38                       |
| 18  | CC3       | T2aN0G3                     | IB                | —               | 72                       |
| 19  | CC4       | T2aN0G3                     | IC                | —               | 72                       |

...Continued

Finally, identification of proteins correlated with genomic instability has the potential to improve malignancy grading.

In the present study, we expand the current knowledge about the expression of proteins in EEC with respect to DNA ploidy as a measure of genomic stability and the relevance of these proteins to EEC carcinogenesis.

MATERIALS AND METHODS

Clinical material

Clinical material (Table 1a and b) was collected at the Department of Obstetrics and Gynecology, Karolinska University Hospital, Huddinge; the Department of Gynaecologic Oncology, Radiumhemmet, Karolinska University Hospital, Solna, Sweden; and the Department of Oncology and Medical Radiology, Lviv National Medical University, Lviv, Ukraine, with informed consent and approval from the local ethics committees (Stockholm County Council – Dnr. 97-244 (1998-03-02), 00-068 (200-06-05), 2006/649-31/4, Ethics Committee of Lviv National Medical University – protocol no. 2).

Tissue biopsies of EEC (15 cases), SCC (13 cases) and control tissue from patients with nonmalignant gynaecological diseases (e.g., myoma and menorrhagia) consisting of normal endometrium and the latter in 6 mm thick tissue cuts. The prepared slides were stained according to the Feulgen method and the DNA content in

DNA cytometry

Tissue biopsies of EEC (Table 1a) and an independent group of FFPE samples of EEC and AH (Table 1b) were analysed for DNA ploidy. The former were analysed in imprint cytological samples and the latter in 6 mm thick tissue cuts. The prepared slides were stained according to the Feulgen method and the DNA content in...
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All steps were performed as previously described (Lomnytska et al., 2010). Protein expression of protein spots was analysed by Progenesis SameSpot software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Protein biomarkers of genomically unstable endometrial cancer

Figure 1 Description of the clinical material used in this study. (A) DNA histograms of diploid stable EEC showing narrow stem line in the 2c region, diploid unstable EEC with a broad stem line that expands from the 2c to the 4c region and aneuploid unstable EEC with a broad peak outside the 2c region and additional peaks exceeding the 4c region. (B) Examples of analysed 2D gels of EEC and endometrium. (C) Principal component analysis of the analysed 2D gels indicating similarity between the expression of protein spots in genomically unstable EEC and SCC, genomically stable EEC and normal endometrium as well as difference between the expression of protein spots in genomically stable and unstable EEC. (D) Clustering of identified proteins according to their function with numbers corresponding to the amount of detected proteins. (E) Distribution of selected proteins according to gains (to the right) and losses (to the left) on the chromosomes where the orange colour corresponds to early chromosomal changes during EEC carcinogenesis. A shaded pattern depicts chromosomal changes related to a bad prognosis for patients.

Two-dimensional gel electrophoresis and MALDI TOF mass spectrometry

Tissue proteins were extracted and solubilised in lysis buffer: 9 M urea (Bio-Rad, Sundbyberg, Sweden), 2 M thiourea (USB, Cleveland, OH, USA), 5% Resolyte (BDH, Poole, Dorset, UK), 65 mM DTT (Bio-Rad), 1 mM EDTA (Merck, Darmstadt, Germany), 0.5% w/v Nonidet P-40 (USB), 25 mM CHAPS, 0.1% PMSF, 0.01% benzamidine, 0.01% BHT, and 35 mM NaOH (Sigma, St Louis, MO, USA) (Hellman et al., 2009). Protein concentration was determined using the Bradford protein assay (Bradford, 1976). The IEF, SDS–PAGE, staining with silver nitrate and excision of spots were performed as described before (Lomnytska et al., 2010). Expression of protein spots was analysed by Progenesis SameSpot software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Protein spots with a relative expression difference of 1.5-fold (ANOVA with \( P < 0.05 \) and power > 0.8) were selected for MALDI TOF MS. All steps were performed as previously described (Lomnytska et al., 2010).

Western blot

In order to verify the identity of the proteins after MALDI TOF MS analysis, the same tissue protein lysates that were used for 2D gel analysis (Figure 2A) were subjected to western blot (Figure 2B). Equal concentration of protein lysates was applied to 10.5–14.0% SDS–PAGE (Criterion gels, Bio-Rad). The following commercial antibodies were used for western blot: EIF4A1 (1:2000; ab31217-100, rabbit polyclonal; Abcam, Cambridge, UK), CLIC1 (1:500; ab77214-100, mouse monoclonal; Abcam), PRDX6 (1:4000; ab59543, rabbit polyclonal; Abcam), ANXA4 (1:1000; ab109900, rabbit polyclonal; Abcam), ANXA4 (1:1600; ab109900, mouse monoclonal; Abcam), EMD (1:1000; ab54996, mouse monoclonal; Abcam) and Ku70 (1:1000; S5C11, mouse monoclonal; Abcam). All antibodies were diluted in Pierce (Rockford, IL, USA) Protein-Free T20 (PBS) Blocking Buffer (Thermo Scientific, Middletown, VA, USA) and incubated for 12 h at 4°C. As positive controls, lysates of cell lines that contain corresponding antigens were used, that is, HeLa cell lysate for EIF4A1, CLIC1, PRDX6 and EMD, placenta lysate for ANXA4 and MCF7 cell lysate for CLIC4, ENO1 and Ku70 (Figure 2B). The membranes were incubated in secondary antibody of the corresponding species, diluted 1:15000 in Pierce Protein-Free T20 (PBS) Blocking Buffer for 1.5 h at room temperature, followed by 4 washes of 15 min in PBS-T. Finally, the proteins were visualised by ECL. The secondary antibodies used were HRP-linked anti-mouse (NXA931) and HRP-linked anti-rabbit antibodies (NA934VS, GE Healthcare, Chalfont St Giles, UK). All steps were performed as described before (Lomnytska et al., 2010).
Immunohistochemistry

An immunohistochemical analysis was carried out on FFPE samples of EEC, AH and E of an independent group of patients in order to study the expression of the identified proteins during EEC carcinogenesis (Table 1b). Immunohistochemistry was performed using the two-step streptavidin–biotin method. Tissue slides were incubated overnight with the primary antibodies in 1% BSA at 4°C. Antibodies used previously for western blot were applied in following dilutions for IHC: EIF4A1 (1 : 200), CLIC1 (1 : 10), PRDX6 (1 : 1000), ENO1 (1 : 200) and Ku70 (1 : 400). In addition, staining against ANXA4 (1 : 200; sc-1930, goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CLIC4 (1 : 30; HPA008019, rabbit polyclonal; Sigma-Aldrich, St Louis, MO, USA) and EMD (1 : 300; HPA000609, rabbit polyclonal; Sigma-Aldrich) was performed (Figure 3A). Antibodies used for western blot against ANXA4, CLIC4 and EMD were also used for IHC for confirmation of specificity (data not shown). Several visualising systems were used: VectaStain (Vector, Peterborough, UK) ABC-Po-kit and DAB (positive stain was brown), LSAB+ (DAKO, Glostrup, Denmark) (positive stain was red). Control tissues that contained corresponding antigens were also utilised: placenta tissue for ANXA4, placenta and tonsillar tissue for EIF4A1, tonsillar and ovarian tissue for CLIC1, tonsillar and placenta tissue for PRDX6, tonsillar, placenta and breast cancer tissue for CLIC4, colon cancer and tonsillar tissue for EMD, breast cancer and tonsillar tissue for Ku70 and breast cancer and kidney tissue for ENO1. Images were captured with a Leica DM4500B (camera DFC320, ocular 10 ×, objectives 20 ×/0.50 HC PL and 40 ×, 506145) and the Leica Application Suite software, version 2.4.0 (Wetzlar, Germany) as 16-bit depth .tif format images with 48-bit image resolution, and expression of the analysed proteins was scored as previously described (Cheng et al, 2008; Lomnytska et al, 2011).

Statistical analysis

We used the inbuilt statistical chapter of SameSpot Nonlinear software (PCA, ANOVA, power, t-test), MedCalc, version 11.1.1.0 (Mariakerke, Belgium) (receiver-operator-characteristic (ROC) curves) and Statistica 6.0 (Tulsa, OK, USA), (correlation, t-test, χ2 test). A difference of P<0.05 was considered statistically significant.

RESULTS

Expression of protein spots in analysed 2D gels

A total of 42 2D gels were generated from tissue biopsies of 40 patients with EEC, SCC or nonmalignant gynaecological diseases (Table 1a), with each gel containing ~2000 protein spots (Figure 1B). DNA cytometry was performed on all EEC samples in order to identify their genomic stability (Table 1a and Figure 1A).
Based on the DNA pattern, EEC cases were divided into two major groups – genomically stable EEC that included five diploid stable cases and two aneuploid stable cases and genomically unstable EEC that consisted of seven diploid unstable cases and two aneuploid stable cases and genomically unstable endometrioid endometrial cancer as evaluated by receiver-operator curves. (C) Sensitivity and specificity for discrimination between (a) endometrium and atypical hyperplasia of endometrium, (b) endometrium and genomically unstable endometrioid endometrial cancer, and (c) atypical hyperplasia of endometrium and genomically unstable endometrioid endometrial cancer as evaluated by receiver-operator curves.

Function relevance of the identified proteins

Functional activity of the identified proteins was analysed using the NCBI/Protein and OMIM databases. We divided the proteins in the major functional groups, that is, regulators of cell cycle and apoptosis, migration and adhesion, metabolism, transcription and translation, maintainers of DNA, members of extracellular matrix and scaffold proteins. We observed that the representation of the proteins that were over- or under-expressed in EEC in the studied functional groups was unequal (P = 0.0006; Figure 1D).

Verification of protein identification

In order to confirm the accuracy of the protein identification with MALDI TOF MS, the tissue protein lysates used for 2D gels were immunoblotted using commercially available antibodies against eight selected proteins: EIF4A1, CLIC1, PRDX6, CLIC4, ENO1, ANXA4, EMD and Ku70.
ANXA4, EMD and Ku70 (Figure 2). Expression of the protein spots in 2D gels of two selected cases of EEC and two controls is shown (Figure 2A). Concomitantly, their expression was verified using western blot for these respective cases (Figure 2B), confirming their expression profile in EEC. The expression profile of EIF4A1 and ANXA4 could not be confirmed by western blot but, conversely, on 2D gels their observed molecular weight was lower than expected. This could be because of cancer-specific over-expression of truncated forms of these proteins.

Expression of EIF4A1, CLIC1, PRDX6, CLIC4, ENO1, ANXA4, EMD and Ku70 in genomically stable and unstable EEC, AH and endometrium as evaluated by IHC

As these identified proteins have not been previously analysed in connection with EEC, their expression was investigated in greater detail. Therefore, a set of independent cases was subjected to IHC, encompassing normal endometrium, AH, a precursor lesion of EEC, and genomically stable and unstable EEC (Table 1b). According to the IHC analysis, expression of EIF4A1 and CLIC1 increased in the nuclei of atypical cells and cytoplasmic expression of PRDX6 was enhanced in AH and genomically unstable EEC. A tendency towards decreased cytoplasmic expression of CLIC4 was observed in genomically stable and unstable EEC. Although ENO1 was not significantly overexpressed in 2D gels of EEC (Table 3 and Figure 2A), its cytoplasmic expression was low in AH and genomically unstable EEC (Figure 3B). Also, low cytoplasmic expression of ANXA4 was observed in genomically stable and unstable EEC (Figure 3B). Interestingly, only the N-terminal part of ANXA4 was significantly overexpressed in 2D gels of EEC. This fragment migrated at 17 kDa whereas the molecular mass of the full-length protein is 34 kDa (Figure 2). Nuclear expression of EMD was low in AH, genomically stable and unstable EEC. Expression of Ku70 was highly abundant in endometrium and low in genomically stable and unstable EEC (Figure 3A and B).

Using ROC curves (Figure 3C), we determined that the expression of CLIC1, EIF4A1 and PRDX6 displayed the highest sensitivity and specificity for discrimination between E and AH (Figure 3Ca). Expression of EMD, Ku70 and ANXA4 depicted the highest sensitivity and specificity for discrimination between E, AH and genomically unstable EEC (Figure 3Cb and c). Thus, we demonstrated that changes in protein expression observed in EEC can already be detected on the level of AH. No statistically significant difference was found between the expression of the proteins in genomically stable and unstable AH.

DISCUSSION

Malignancies are classically divided into diploid and aneuploid based on DNA ploidy. However, it has been shown in breast cancer that further subclassification into stable and unstable diploid and aneuploid tumours provides more accurate prognosis (Kronenwett et al, 2006). Our analysis of the tissue proteome of EEC offered a possibility for re-classification of this malignancy into stable and unstable subtypes. In particular, our analysis of 2D gels did not show any difference between the expression of proteins in diploid and aneuploid genomically unstable EEC, but showed a clear difference with diploid genomically stable EEC. In addition, similarities were observed between protein expression in genomically unstable SCC and genomically unstable ECC, suggesting an impact of genomic instability on protein expression. By comparing EEC and SCC, we identified changes in protein expression specific for EEC while excluding proteins commonly overexpressed in most malignancies (Petrak et al, 2008).

We also confirmed the identity of several proteins previously found to be overexpressed in endometrial cancer. One interesting example was CAPS (Li et al, 2008a), a protein related to low differentiation and worse survival of patients with endometrial cancer (Li et al, 2008b). Among the proteins linked to proliferation and invasion of endometrial cancer (Yi et al, 2009), we identified HSPA1, TPM2, PDIA, ENO and HNRNPK. Among the proteins downregulated in EEC in connection to invasion into myometrium (Monge et al, 2009), we identified MSN (family of EZR), TUBA1B, ANXA1, HNRNP3 and TALDO1. We also observed a high expression of HSP90AA1, PTGES3 and ATP5B in relation to the stage of EEC (Supplementary Table S2).

Our study focussed on the analysis of protein expression in EEC whereas other groups have analysed chromosomal changes in EEC and in AH (Sonoda et al, 1997; Suzuki et al, 1997; Kiechle et al, 2000; Baloglu et al, 2001; Schulten et al, 2004; Levan et al, 2006; O’Toole et al, 2006) and CIN3 and SCC (Heselmeyer et al, 1996, 1997) (Supplementary Table S3 and Figure 1E). Once synthesised, proteins generally undergo numerous post-translational modifications in order to become functionally active. We observed
Table 3: Expression of identified proteins in genomically stable and unstable EEC in comparison with SCC

| Protein spot № | GO | Chromosome |
|----------------|-----------------|----------------------------------|
| 1              |                 | Genomically unstable EEC compared with EEC | Genomically stable EEC compared with SE |
| 2              |                 | Genomically unstable EEC compared with SCC | Genomically stable EEC compared with SCC |
| 3              |                 | Genomically unstable EEC compared with SCC | Genomically stable EEC compared with SCC |
| 4              |                 | Genomically unstable EEC compared with SCC | Genomically stable EEC compared with SCC |
| 5              |                 | Genomically unstable EEC compared with SCC | Genomically stable EEC compared with SCC |
| 6              |                 | Genomically unstable EEC compared with SCC | Genomically stable EEC compared with SCC |

Note:
1. Number of protein spots on 2D gel.
2. One-letter amino acid abbreviations.
3. Genomic instability.
4. In genotypically unstable EEC and endometrium (E).
5. In genotypically stable EEC and endometrium (S).
6. In SCC and squamous cervical epithelium (SCC).

Abbreviations: EEC = endometrioid endometrial cancer; SCC = squamous cervical cancer.
underexpression of ENO1 and CLIC4 in both EEC and AH. Interestingly, loss of a specific part of the 1p chromosome is a key event during EEC carcinogenesis and this deleted region is responsible for the synthesis of ENO1 and CLIC4 (Kiechle et al., 2000; Baloglu et al., 2001). Other early events during EEC carcinogenesis are gains in the entire long arm of the 1q chromosome that contains the gene coding for PRDX6 and losses at 22q chromosome that disrupt the synthesis of Ku70 (XRC6) (Kiechle et al., 2000; Baloglu et al., 2001), which also corresponds to our findings on the protein level in EEC and AH. In addition, EEC is characterised by gains at the 2p, 6p, 17p and Xq chromosomes (Suzuki et al., 1997) and those are responsible for the synthesis of ANXA4, CLIC1, EIF4A1 and EMD, respectively. In contrast to this, we observed decreased expression of ANXA4 in AH and EEC according to our IHC data, whereas we confirmed increased expression on our 2D gels. This discrepancy can be explained by the fact that the molecular weight of ANXA4 detected on the 2D gels was lower than expected and the protein was represented only by the NH2 domain. This can be due to cancer-specific truncation of the NH2 domain, leading to malfunction of the full-length protein (Gerke and Moss, 2002). EMD was also underexpressed in EEC and AH, which corresponds to its functional role in maintaining chromosomal stability.

For the first time, our paper describes EIF4A1, CLIC1, PRDX6, CLIC4, ENO1, ANXA4, EMD and Ku70 in relation to EEC, although their role is well established in other cancers. EIF4A1 is overexpressed in hepatocellular carcinoma (Yoon et al., 2006) and is an early marker of distant metastases of non-small cell lung cancer (Ji et al., 2003). Similarly, we find it overexpressed in AH, suggesting that EIF4A1 expression could also be used as an early marker of EEC. CLIC1 is involved in invasion, cancer cell motility (Wang et al., 2009) and development of chemoresistance (Kang and Kang, 2008). It is overexpressed in nasopharyngeal carcinoma (Chang et al., 2009), colorectal cancer (Petrova et al., 2008) and hepatocellular cancer (Huang et al., 2004). PRDX6 protects against oxidative injury, it is overexpressed in endometriosis (Stephens et al., 2010) and it increases the invasiveness of breast cancer (Chang et al., 2007). CLIC4 is a chloride intracellular channel that translocates to the nucleus in response to DNA damage and is associated with growth arrest and apoptosis. Moreover, loss of the expression of CLIC4 in cells and upregulation in stroma is associated with malignant progression (Suh et al., 2007a,b). ENO1 is a glycolytic enzyme that binds to the promoter of the oncogene c-myc and acts as a transcriptional repressor (Feo et al., 2000). Therefore, we hypothesise that loss of ENO1 leads to increased c-myc expression, which is known to promote carcinogenesis. The transcription and translation of ANXA4 in endometrium is regulated by progesterone, an important regulator of cyclic changes in endometrium (Ponnampalam and Rogers, 2006). EMD belongs to the inner nuclear membrane proteins that bind chromatin modifiers (Shaklai et al., 2007). Its loss in ovarian cancer is considered to be the basis for aneuploidy (Capochichi et al., 2009). Ku70, or XRC6, is a nuclear complex involved in the repair of double-strand non-homologous DNA breaks. Malfunction of the XRC6 gene is observed in ovarian cancer (Kim et al., 2010) and breast cancer (Willems et al., 2009).

In summary, we analysed the tissue proteome of EEC with respect to genomic stability, one of the most important prognostic markers (Lundgren et al., 2002, 2004), and identified differentially expressed proteins. We showed that changes in protein expression could already be detected in precursor lesions, that is, atypical hyperplasia of endometrium, which could provide significant improvement in early detection of EEC.

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Conflict of interest

The authors declare no conflict of interest.

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