Proteomic analysis of a preneoplastic phenotype in ovarian surface epithelial cells of prophylactic oophorectomies

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Abstract

Objective. To study the pattern of protein expression associated with the early molecular changes in ovarian carcinogenesis.

Methods. Prophylactic oophorectomy is used to prevent ovarian carcinoma in high-risk populations who have a strong family history of breast/ovarian cancer. The ovarian surface epithelium (OSE), which is tissue of origin of epithelial ovarian cancer, of these ovarian specimens often contains altered morphology, growth patterns, and differentiation features that are believed to be preneoplastic. This study has used a proteomic approach, based on two-dimensional gel electrophoresis and mass spectrometry, to compare the protein profiles of OSE from women with a history of familial ovarian cancer (FH-OSE), i.e. at least two first-degree relatives with such cancer and/or testing positive for *BRCA1* mutations, to those without such history (NFH-OSE).

Results. Of >1500 protein spots, 8 proteins whose levels were significantly altered in FH-OSE. Three were known ovarian-tumor associated proteins, others were novel changes. A number of the alterations seen were also accompanied with protein modifications, and have not been previously reported. There was a predominance of sequences related to the stress response pathway. Differential expression of selected genes was confirmed by Western blotting and real time reverse transcription polymerase chain reaction.

Conclusions. Our findings define the OSE phenotype of women at a significant high risk of developing ovarian cancer. Protein alterations seen in these (pre)neoplastic tissues may represent an early, irreversible, non-mutational step in ovarian epithelial neoplastic progression, and may be

potential early and sensitive markers for the onset of transformation.

Introduction

The epithelial ovarian carcinomas, which originate from the ovarian surface epithelium (OSE), are the prime cause of death from gynecological malignancies in European and North American women. One reason for the high mortality of ovarian cancer is that, unlike many other cancer types, ovarian cancer is notorious for its insidious properties in early stages. Close to 70% of patients present with the disease spread beyond the pelvis, resulting in a long-term survival rate of only 29% [1]. Indeed, if a woman is diagnosed with an early stage (stage I) ovarian cancer, the survival rate is close to 90% without altering current therapeutic approaches. Thus, it is urgently needed to understand the early events and etiology of the disease. Although there are many genetic and environmental factors which can influence a woman's risk of getting breast and ovarian cancer, a strong family history is by far the most important and best-defined epidemiological risk factor.

Recently, cancer-prone women with an inherited predisposition to ovarian cancer, often *BRCA1* (a candidate tumor suppressor in breast and ovarian carcinomas) mutation carriers, underwent prophylactic oophorectomy as a preventive approach [2, 3]. The high prevalence of ovarian cancer in cancer-prone women provides an excellent model to uncover new players in early ovarian carcinogenesis and, perhaps, means of detecting ovarian cancer at an early, curable stage. Importantly, several studies have identified microscopic benign-to-malignant morphologic features in these ovarian specimens, suggesting the existence of preneoplastic phenotypes in the cells [4-7]. However, little is known about the molecular changes that are associated with or account for the preneoplastic morphologic changes.

Over 90% ovarian cancers are thought to arise from the ovarian surface epithelium (OSE), which is a simple epithelial layer covering the ovaries. Since OSE is a minute part of the intact ovary, only limited amount of tissue can be obtained from a single specimen. Thus, the ability to culture OSE provides an opportunity of obtaining large enough quantities of relatively pure populations of ovarian epithelial cells for *in vitro* studies [8, 9]. Although several previous studies have identified genes differentially expressed in ovarian cancer, our analysis represents a better approximation of the earliest stage of ovarian cancer development. The majority of previous analyses were performed in ovarian cancer cells and compared with normal OSE cells [10-12], whereas we chose to compare overtly normal OSE cells of high-risk individuals to OSE cells of the general population. We and others have revealed the presence of premalignant histologic and/or biologic alterations in OSE cells from prophylactically removed ovaries of high-risk individuals [13-17].

This study has used a proteomic approach to compare the protein profiles of OSE from high-risk individuals with those of the general populations. This powerful analytical technology, in contrast to array methodologies as in previous comparative gene studies, is able to provide an unbiased and comprehensive expression profiling without prior knowledge of the expressed proteins in the starting material [10-12]. It also offers the advantages of detection at the functional level of protein expression and the ability to also detect posttranslational modifications of proteins, which can easily be missed by transcriptional profiling. Differential expression of selected genes observed in this study was further confirmed by Western blotting and real time reverse

transcription polymerase chain reaction.

Materials and methods

Cell cultures

Experimentation with human tissues was approved by the University's ethics committee prior to this study and the normal human OSE samples collected were made anonymous. Briefly, NFH-OSE (OSE-29, OSE-80 and OSE-398) cells were obtained from women in the general population with no family history of breast/ovarian cancer, having surgery for non-malignant gynecological diseases. FH-OSE (OSE-229F, OSE-261F and OSE-267F) were obtained from women who underwent prophylactic oophorectomy because of strong family histories of breast/ovarian cancer, i.e. at least two first-degree relatives with such cancer, and/or testing positive for *BRCA1* mutations. *BRCA1* site specific mutation analysis revealed *BRCA1* del 185 AG for OSE-261F, and *BRCA1* 3867 G>T for OSE-267F. The genetic analysis of OSE-229F was incomplete. To obtain enough quantities of OSE cells, IOSE ("immortalized OSE") lines were generated by transfecting OSE cells in passages 2-3 with the SV40 early genes (large and small T antigen) to extend their lifespan in culture, but remained nontumorigenic [18]. Cultures were maintained in a 1:1 mixture of 199/MCDB 105 medium (Sigma, St. Louis, MO) supplemented with 5% FBS (Hyclone Laboratories Ltd., Logan, UT) in 5% CO_2 - 95% air, and passaged using 0.06% trypsin/0.01% EDTA (Invitrogen, Carlsbad, CA) [8, 9].

2-D gel electrophoresis

The 2-D gel electrophoresis was carried out with Amersham Biosystems IPGphor IEF and Ettan Dalt six electrophoresis units by following the protocol described previously [19]. Protein samples (~ 100 µg) were applied to the 2-D gel electrophoresis (13 cm) and run in pair side by side. Briefly, ~100 µg of proteins extracted from cells were mixed up to 250 µl of rehydration solution containing 8 M Urea, 4% CHAPS, 1 mM PMSF, 20 mM DTT and 0.5% IPG buffer. The rehydration step was carried out with precast 13 cm IPG strips for more than 10 hr at low voltage of 30V. IEF was run following a step-wise voltage increase procedure: 500V and 1000 V for 1 h each and 5000-8000 V for about 10 h with a total of 64 KVh. After IEF, the strips were subjected to two-step equilibration in equilibration buffers (6 M Urea, 30% Glycerol, 2% SDS and 50 mM Tris-HCl pH 6.8) with 1% DTT (w/v) for the first step, and 2.5% Iodoacetamide (w/v) for the second step. The strips were then transferred onto the second-dimensional SDS-PAGE that was run on 1.5 mm thick 12.5% polyacrylamide gels at 10˚C. Triplicate electrophoresis was performed to ensure reproducibility. All gels were visualized by silver staining [19].

Image analysis and MS peptide sequencing

Image acquisition was performed with ImageScanner and analyzed with ImageMaster 2D Elite software (Amersham-Pharmacia Biotech, Piscataway, NJ). Comparisons were made between gel images. Each spot intensity was processed by background subtraction and total spot volume normalization to compensate the variation of protein loading. Normalized volume differences were

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statistically calculated. Consistently and significantly different spots were selected for analysis with MALDI-TOF mass spectrometry. Protein spots were cut off in small pieces and subjected to in-gel tryptic digestion. Peptide mass spectra were recorded and parameters for spectra acquisition were used as stated previously [18]. In database protein matching using MS-Fit (http://prospector.ucsf.edu), 25 ppm or less mass errors and MOWSE scores over 300 were obtained in most of analyses. Duplicate or triplicate runs were made to ensure an accurate analysis.

Western Blot

Protein lysate (10 μg) was separated on 12% SDS-PAGE, and then transferred onto a PVDF membrane. After blocking with 5% non-fat milk at $4^{o}C$ overnight, the membrane was probed by primary antibodies to BiP, rabbit polyclonal antibody (1:1000) (no. SPA-826; Stressgen, Victoria, B.C., Canada); GRP94, rat monoclonal antibody (1:1000) (no. SPA-850; Stressgen, Victoria, B.C., Canada); annexin A11, goat polyclonal antibody (1:500) (no. sc-9321; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and tropomyosin, mouse monoclonal antibody (1:1000) (no. T2780; Sigma, St. Louis, MO) for 2 hours at room temperature or overnight at $4^{o}C$ with shaking. After washing, species-specific horseradish peroxidase-conjugated secondary antibody was added for 1 hour at $4^{o}C$, and the antigen-antibody interaction was finally detected by ECL detection kit (Amersham-Pharmacia Biotech, Piscataway, NJ) and then exposed to the x-ray film.

Real time reverse transcription polymerase chain reaction (QRTPCR)

Total cellular RNA was extracted from the cells using Trizol and reverse-transcribed by Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Subsequently, the cDNA (0.5 µl aliquot) was amplified by QRTPCR with the double-stranded DNA specific dye SYBR green using an iCycler real-time PCR detection system (Bio-Rad, Hercules, CA) following manufacturer's instructions. The primers used were: BiP, 5'-CTGGGTACATTTGATCTGACTGG-3' and 5'-GCATCCTGGTGGCTTTCCAGCCA-3'; GRP94, 5'-GCTCAATTGGATGAAAGATA-3' and 5'-GTTTTCTTCTGACTTGCATAG-3'; transgelin, 5'-CGAAGTGCAGTCCAAAATC-3' and 5'-CTGGTTCTTCTTCAATGGG-3', and β-actin, 5'-TCACCGAGGCCCCTCTGAACCCTA-3' and 5'-GGCAGTAATCTCCTTCTGCATCCT-3'. The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. PCR fragments were cloned and sequenced to confirm the corresponding sequence. Relative mRNA expression was determined by dividing the threshold of each sample by the threshold of the internal control β-actin [20]. These experiments were carried out in replicate and independently repeated at least two times.

Results

Proteomic analysis

Fig. 1 shows typical silver-stained 2-D gel images for FH-OSE (IOSE-267F) and control NFH-OSE (IOSE-80) side by side. Around 1500 spots were detected on each gel, ranging from 6-200 kDa in size and 4-10 in p*I*. Normalized spot-volume comparison was made with the assistance of image analysis software (ImageMaster). Six areas where significant and consistent

spot changes occurred in all cases of FH-OSE when compared to the gel image of control NFH-OSE were circled. In some cases, isoforms of proteins were found in spot trains, indicating that modified proteins or isoforms were well separated by 2-D gel electrophoresis.

Protein identification

Based on the image analysis, protein spots that had significant visual differences in size and/or volume were cut off for trypsin digestion, MALDI-TOF mass spectral measurement and database searching. Isoforms that gave an identical primary structure in the protein matching were classified as one protein. Table 1 summarizes the peptide fingerprinting results, there were six proteins (or isoforms) were upregulated and two proteins were suppressed significantly in FH-OSE than the NFH-OSE controls. Most of the matched proteins had high sequence coverage, mass accuracy and MOWSE scores. The fold differences in protein expressions from FH-OSE were indicated, and the proteins were grouped for known functions. Two of the spots, # 108 & 535, belong to protein fragments, as indicated by their appearances at gel regions with molecular weights lower than their intact molecules, 94-kDa glucose-regulated protein (GRP94) and mesoderm induction early response 1, respectively. This identification was also consistent with the fact that matched peptides are concentrated on certain parts of the protein sequences.

Protein alterations

Fig. 2 shows the alterations in the expression levels of the identified proteins between

FH-OSE and NFH-OSE. More proteins showed increased expression than decreased expression in FH-OSE. The actin cross-linking protein transgelin (+3.2- to 5-fold); BiP protein (+1.7- to 3.4-fold) and GRP94 (+1.4- to 3.5-fold), chaperone proteins; annexin A11 (+2- to 7.7-fold), key components of the membrane trafficking; enoyl-CoA hydratase (+1.3- to 3.2-fold), the metabolic enzyme; and mesoderm induction early response gene (+1.6- to 56.9-fold) were upregulated, whereas the expression level of triosephosphate isomerase 1 (-1.1 to -1.6-fold) and tropomyosin (-1.8- to -2.1-fold) was significantly decreased.

Validation of proteomic data on selected candidates

Western blotting was carried out to confirm the 2-D electrophoresis results on four proteins, tropomyosin, annexin A11, BiP and GRP94, because their altered expressions were known to be associated with cell transformation events and thus of particular interest to this study to examine cancer-related changes and antibodies suitable for immunoblotting were available. In all cases, protein expression trends were consistent with those of the 2-D gels. For GRP94, the over-expression of the 50-kDa fragment identified in 2-D gels was confirmed in Western blotting (Fig. 3). The expression levels of β-actin, a housekeeping gene, were shown to be expressed at a constant level. To verify the differentially expressed genes in FH-OSE, expression levels of three genes (BiP and GRP94, in the chaperone stress pathway, and the actin cross-linking protein transgelin) were further confirmed using QRTPCR. As shown in Fig. 4, elevated expression of BiP,

GRP94 and transgelin were confirmed in all FH-OSE lines.

Protein phosphorylation alterations

MALDI-TOF MS spectra were also subjected to possible phosphorylation analysis. Our data suggested that several proteins may have such post-translational modifications at certain sites. For instance, peptide mass fingerprinting indicated possible phosphorylations at T201 or S209 in tropomyosin, S106 in annexin A11, T148/153 and/or Y157 in BiP and T449 or T453 in triosephosphate isomerase 1 (Table 2).

Discussion

Ovaries from cancer-prone women who underwent prophylactic surgery provide an excellent opportunity to identify preneoplastic alterations or early molecular changes in ovarian carcinogenesis, because these women are at a significant risk for developing ovarian cancer compared to women in the general population. The findings here represented the most comprehensive studies to date, confirming and extending previous histologic results indicating potentially preneoplastic features in ovaries from cancer-prone women with an inherited predisposition for ovarian cancer than in control ovaries [4-7]. Compared to studies using the whole ovary, the use of OSE cells in this study adds further confirmatory evidence as to the differences occurring predominantly between normal and preneoplastic epithelial cells in the earliest stage of ovarian cancer. Since the ovaries or OSE cells removed prophylactically from

high-risk women often appear macroscopically normal, our results that only few differentially expressed proteins are identified and the relatively small magnitude of changes between FH-OSE and NFH-OSE samples, as compared to between tumor and normal samples, are therefore can be anticipated. The identification of early molecular changes in OSE cells of high-risk individuals is encouraging, as this contributes to our understanding about the biology of ovarian tissues in women at increased risk of developing ovarian cancer, which to data is still largely unknown.

The most striking finding in this study is the upergulation of proteins involved in protein synthesis and processing such as mesoderm induction early response gene and two members of the chaperone family (BiP and GRP94) were observed in FH-OSE cells as compared to NFH-OSE. The mesoderm induction early response gene encodes a nuclear protein that functions as a transcriptional activator, and has shown to be upregulated in breast carcinoma cell lines and breast tumors when compared to normal breast cells [21]. BiP and GRP94 are chaperones resident in the endoplasmic reticulum (ER), which facilitate protein folding and could limit damage in normal tissues and organs exposed to ER stress [22]. The potential significance of chaperone proteins in ovarian cancer has been illustrated by the high degree of overexpression of BiP and GRP94 in human malignant ovarian ascites fluid [23], supporting the potential involvement of chaperones in ovarian oncogenesis and it is significant that these proteins were identified in this study. In addition, we found that a low Mr GRP94 (~50 kDa) form was significantly overexpressed in OSE of high-risk individuals. The smaller form of GRP94 is likely to be a splice-variant on the basis of RT-PCR and sequencing of this region confirmed the EST sequence, and has not been previously

reported in other normal tissues or malignant tumors. Thus, the expression of the smaller form of GRP94 may therefore suggest important physiological and pathological implications specific to ovarian epithelial cells. Overexpression and antisense data show that these proteins can protect cells against cell death, and the anti-apoptotic function has been hypothesized to be beneficial to situations that lead to cancer progression and drug resistance [22]. Thus, it is plausible that increases in BiP and GRP94 of high-risk individuals may contribute to ovarian epithelial neoplastic transformation by enhancing the survival capabilities of OSE cells in stress-induced cytotoxicity.

Enoyl-CoA hydratase, which is a short-chain mitochondrial enzyme involved in β-oxidation, showed an increased expression in FH-OSE cells as compared to NFH-OSE. Altered expression has also been observed in human colon carcinomas and hepatocellular carcinomas, which are known to frequently exhibit clear-cell or fatty changes [24, 25]. In addition, other pathways including the glycolytic enzyme triosephosphate isomerase was also found to be altered. Together, these findings indicate a wider role for impaired metabolic function in ovarian tumorigenesis, although future work is needed to confirm and investigate this further.

Transgelin was identified previously as a transformation and shape change-sensitive actin-gelling protein [26], and its activity has been found to be suppressed in many cancer cells, including breast and colon cancer cells [27]. Surprisingly, however, we found that expression of transgelin was significantly upregulated in OSE from high-risk individuals when compared to normal OSE. This unexpected finding can in fact be explained by the pathological phenomenon specific to the development of ovarian cancer. In the course of neoplastic progression, unlike

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carcinomas that arise from most tissues that lose differentiation, OSE tend to acquire new and more complex epithelial differentiation that mimics characteristics of the specialized epithelia of Mullerian duct origin, *viz.* the oviduct, endometrium and endocervix [28]. The high frequency of Mullerian characteristics in epithelial ovarian neoplasms suggests that this particular phenotype confers a selective advantage on the transforming of OSE cells. It has been proposed that these changes represent a critical early or even predisposing step towards the ovarian tumorigenicity.

Another actin binding protein with altered expression in FH-OSE was tropomyosin. Tropomyosin is a cytoskeletal microfilament binding protein expressed in both muscle and nonmuscle cells and its expression is known to be associated with malignant transformation. Decreased expression of tropomyosin has been commonly observed in malignant tumors, including breast and ovarian cancer [29, 30]. Interestingly, we found that in addition to the substantial suppression of tropomyosin, this protein may be also constitutively phosphorylated. Other proteins with similar post-translational modifications observed are triosephophate isomerase 1, BiP and annexin A11. The constitutive phosphorylation of these proteins may provide new indications of possible roles for these modifications in the regulation and response of these proteins. It is noteworthy that the carboxyl-terminus domain of BRCA1, a candidate tumor suppressor in ovarian carcinoma, has recently been identified as a protein module that binds to phosphopeptides [31, 32]. Further studies are warranted to understand the phosphorylated targets of BRCA1 and to explain the observed differences.

In summary, our findings have confirmed and extended previous studies suggesting

preneoplastic changes in OSE of high-risk individuals. Many of the findings also illustrate aspects of the underlying pathogenesis, for example, the coordinate increased expression of mesoderm induction early response gene and several chaperone proteins including BiP and GRP94. The antiapoptotic function of these proteins in response to cellular damage or stress has been hypothesized to be beneficial to cancer progression. Moreover, our data identified novel expression profiles and modification trends that are likely to open up new avenues for future studies. The simultaneous analysis of these proteins may be useful for diagnostic purposes for high-risk women to better define their risk of developing ovarian carcinomas.

Acknowledgements

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Figure Legends

Fig. 1. Representative silver-stained 2-D gel electrophoretic protein profiles of NFH-OSE (IOSE-80) and FH-OSE (IOSE-267F). The differences in spot intensities can be visually appreciated. Six areas where significant and consistent spot changes occurred were circled. Molecular weight markers are indicated on the left (in kDa) and approximate isoelectric point (pI) is indicated across the bottom of the gels.

Fig. 2. Cropped images from two-dimensional gels demonstrating differential expression of proteins listed in Table 1. Images from gels of NFH-OSE are on the left, and images of FH-OSE are on the right. *Arrows*, indicate the spots of interest. Spot numbers and protein IDs are indicated in Table 1.

Fig. 3. Confirmation of 2-D gels proteomic trends by Western blot analysis. Immunoblotting of NFH-OSE (N) and FH-OSE (F) using antibodies to tropomyosin, annexin A11, BiP and GRP94 with arrows indicating the proteins of interest. β-actin was used to control the variation in protein concentration.

Fig. 4. QRTPCR measurements of mRNA expression of BiP, GRP94 and transgelin. (a) cDNA prepared from NFH-OSE (N) and FH-OSE (F) were used as templates for PCR using primers

specific for BiP protein, GRP94 and transgelin. β-actin was used to control the variation in mRNA

concentration in the RT-reaction. QRTPCR data were normalized to the expression of β-actin and

reported as relative expression values. All genes showed a significant increase in mRNA

expression in FH-OSE. * $P < 0.05$, ** $P < 0.01$. (b) QRTPCR products were analyzed on a 1%

agarose gel.