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13. ABSTRACT (Maximum 200 Words)

One of the major clinical challenges in the treatment of ovarian cancer is that the cancer cells are, or become, resistant to the drugs used to treat the disease. When the cell no longer responds to the drugs, the cancer continues to grow unabated. Some cellular factors that contribute to making a cell resistant to chemotherapy drugs have been identified, though many still remain to be discovered. These cellular factors or proteins involved in drug resistance can be measured using sensitive analytical techniques. A. major goal of the research proposed in this study is to analyze these proteins from ovarian cancer cell lines that are known to be either sensitive or resistant to the chemotherapeutic drug cisplatin, a first line treatment for ovarian cancer. We will determine if there is a specific protein "fingerprint" that is indicative of either sensitivity or resistance to cisplatin. Once the useful factors that influence drug resistance are identified in cell lines and verified using tumor biopsies, we anticipate that this information could then be used to help predict whether a specific tumor will respond to a specific treatment. To date, the sensitivity of a specific ovarian carcinoma to a specific treatment can only be assessed by administration of the treatment and then observing the outcome. Knowing the factors that contribute to a cancer being sensitive or resistant and having the methods to determine if these factors are present or absent in a given tumor are the goals of this proposal. This information could then be used in the clinical assessment to determine the best course of treatment for a specific cancer.

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Introduction

A major impediment to successful treatment of ovarian cancer is clinical resistance to chemotherapeutic agents (Perez et al., 1991). In addition, despite recent advances (Mills et al., 2001; Srinivas et al., 2001), difficulties in detection of ovarian cancer result in most women presenting with advanced disease (Boente et al., 1996). Diagnosis of resistant or refractory cancer relies almost solely on administration of the treatment and observation of the clinical outcome. The research described in this proposal directly addresses each of these issues relevant to ovarian cancer. Considering that combinational therapy which includes cisplatin is a first-line treatment for ovarian cancer (McGuire and Ozols, 1998), our work will focus on cisplatin resistant ovarian cancers.

Body

Specific Aim1 Task 1

Our original statement of work presented two specific aims of which one was to be accomplished in the first year of the grant period. We will report on our progress to date on the first specific aim and highlight the results of the experiments from each of the proposed tasks in the approved statement of work. The first specific aim was to "develop the methodologies to allow the analysis of proteins with an affinity for cisplatin-damaged DNA to be identified via proteomics technology." There were 2 tasks proposed each with 4 sub tasks. The first task involved development of affinity matrices for retention of proteins with an affinity for cisplatin-damaged DNA. We successfully developed a Sepharose based matrix and optimized the substitution of the cisplatin-damaged DNA by modifying the methods for preparation of the DNA. With greater degree of substitution of the DNA on the matrix, selectivity was increased and we can retain a large number of proteins specifically on the matrix and elute them in a small volume under relatively mild conditions. The results presented in figure 1 are a representative single dimension SDS PAGE analysis of cell free extracts prepared from ovarian cancer cells fractionated on the Cisplatin-damaged DNA Sepharose matrix. An aliquot of proteins loaded on the column is shown in lane 1 with the proteins that were not specifically bound eluted in fractions 2-4. The proteins specifically retained on the matrix were eluted with 0.4 M NaCl and are presented in lanes 5-7 and reveal a series of proteins ranging from 20-200 kDa. The first elution, lane 5 contains 90% of the eluted protein and was selected for further analysis. The efficiency of elution and the concentration of the eluted protein obtained are more that adequate for mass spectrometry analysis and we did not pursue the microlatex bead based affinity matrix.

The second subtask of Task 1 was to optimize the SELDI-TOF MS analysis of the fractionated ovarian cancer cell extract. The first elution fraction was used and protein spotted directly on a gold chip for analysis. Different energy absorbing matrices were tested and the results revealed that SPA consistently out performed CHCA and therefore is used unless noted otherwise. Samples of the eluted proteins were also processed for both solution and on chip trypsin digestion as detailed in the statement of work. The results presented in Figure 2 demonstrate that SELDI-TOF MS analysis of the undigested protein (Panel A) reveals a series of proteins with M/Z values ranging from 5 kDa to 20 kDa, effectively increasing the range protein sizes that can be analyzed. The intensity of the protein peaks, while clearly above background, was not near the maximum that can

be detected. Therefore, to increase sensitivity and detection of proteins that "do not fly as well" in the SELDI-TOF MS trypsin digestion of the eluted proteins was performed. An in solution digestions was performed and the results are presented in Figure 2 panel B. As evident from the intensity and complexity of the spectrum, we have increased the sensitivity and a significant increase in peaks was observed, as expected. These results will allow us to have more determinants in the sensitive and resistant ovarian cancer fingerprint and may also allow the identification of specific proteins present in the protein pool eluted from the affinity matrix.

Specific Aim 1 Task 2.

The second main task to b accomplished in the first year of the grant was to determine the efficiency and utility of direct derivatization of activated SELDI-TOF chips with cisplatin modified DNA to analyze the proteins with an affinity for this DNA structure. We initially prepared DNA substrates with a 5' aminolinker modification. This modified DNA terminus with react with the activated matrix on the SELDI PS10 or PS20 CHIPS which both contain amino reaction chemistries. We compared the efficiency of each of these chemistries for reacting with the 5' aminolinker modified DNA. The results presented in Figure 3 shown the outcome of these analyses. The amount of DNA bound to the chip was determined by reacting a specific amount of DNA quantifying the amount of DNA that did not bind the spot. We employed terminal transferase labeling of the DNA with a radioactive label and separation of the products by DNA sequencing gel electrophoresis. Positive controls labeling know amounts of the substrate are presented in lanes 1-4 and show a clear titration with increasing DNA. The results in the DNA not bound by the PS10 and 20 chips indicate that a significant amount of the DNA was bound by the chips with the PS10 being more efficient than the PS20. The single stranded 30 base DNA substrates used in these analyses indicate a high degree of derivatization of the CHIP and the PS10 was used to analyze protein binding. Considering the nature of the DNA on the chip, a short single stranded DNA, we sought to validate the methodology with a protein known to have a high affinity for the DNA. Replication protein A (RPA) was purified and bound to the derivatized spots. The results obtained demonstrated that RPA binding to the chips was largely independent of derivatization of the spot with DNA. We are able to detect the 14 kDa subunit on spots with and without DNA. When extensive washing of the spots was performed to reduce the DNA independent binding of RPA to the CHIP we also lost binding to the spot derivatized with the DNA. These results indicate that RPA is not binding efficiently to the DNA on the chip. There are two possibilities we considered, first is that the DNA is too short to support binding and the second that the DNA may be bound to the chip by the nitrogenous bases in the DNA and therefore may restrict access to proteins. To overcome these issues we have employed a 5' biotin labeled DNA primer that was used in PCR reactions to amplify a 200 bp duplex DNA that contains a single 5' biotin. This DNA was then bound to a PS10 chip to which we derivatized streptavidin. This procedure effectively establishes a single attachment site for the DNA and a considerable longer DNA length to promote binding of the protein. Considering the nature of this DNA substrate we validated this CHIP methodology using purified Ku protein, which has a high affinity for duplex DNA ends. SELDI-TOF MS analysis is presented in Figure 4

and the results demonstrate that Ku is selectively retained on these spots. We also performed on chip trypsin digestion of the bound Ku and were able to identify numerous peptides of the Ku dimer, which increased the sensitivity of the detection.

Key research Accomplishments

- Established column fractionation procedures for retention and elution of cisplatin-damaged DNA binding proteins.
- Established procedures for SELDI-TOF MS analysis of the eluted proteins including "on chip" and in solution trypsin digestion of the eluted proteins.
- Initiated MS analysis of the cisplatin-damaged DNA binding proteins in the A2780 series of ovarian cancer cells.
- Established methodologies for "on chip" selective retention of DNA binding proteins using streptavidin derivatization of the protein chip and the oriented binding of biotin labeled DNA.
- Established "on chip" trypsin digestion of proteins retained on the DNA modified chip.

Reportable outcomes
None

Conclusions

The research described in this report will enable us to pursue the second specific aim proposed in our grant application and out lined in the approved statement of work. The comparison of the protein expression profiles from cisplatin sensitive and resistant cancer cell lines will provide valuable information and may be useful for predicting the response to certain therapies. In addition, the procedures established may also allow novel determinant of cellular resistance to cisplatin to be identified.

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Appendix
Figures 1-3

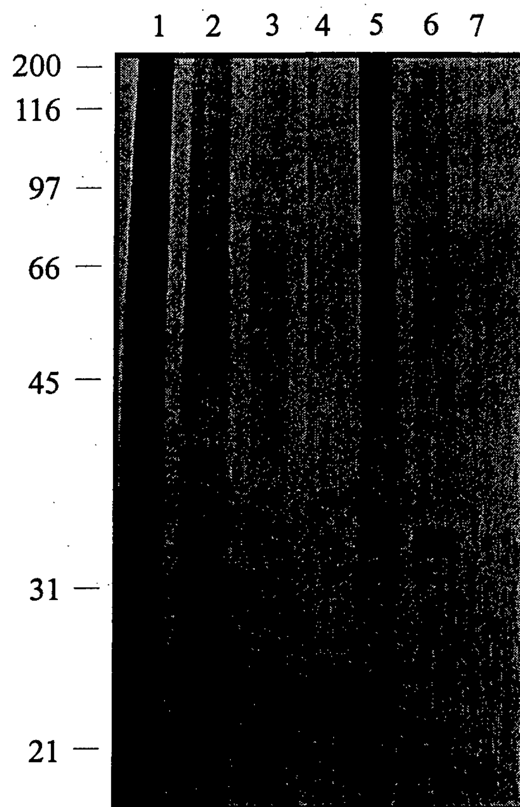
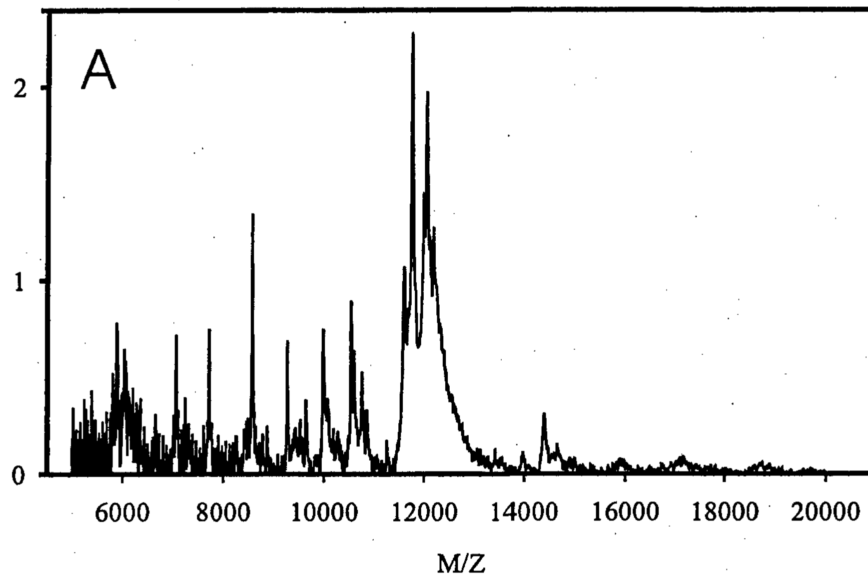


Figure 1. SDS Page analysis of protein fractions from a mini-cisplatin DNA Sepharose column. Aliquots from the cell extract (lane 2), unbound protein (lane 2-4) and specifically bound proteins (lane 5-7) were separated by SDS-PAGE and stained with silver.



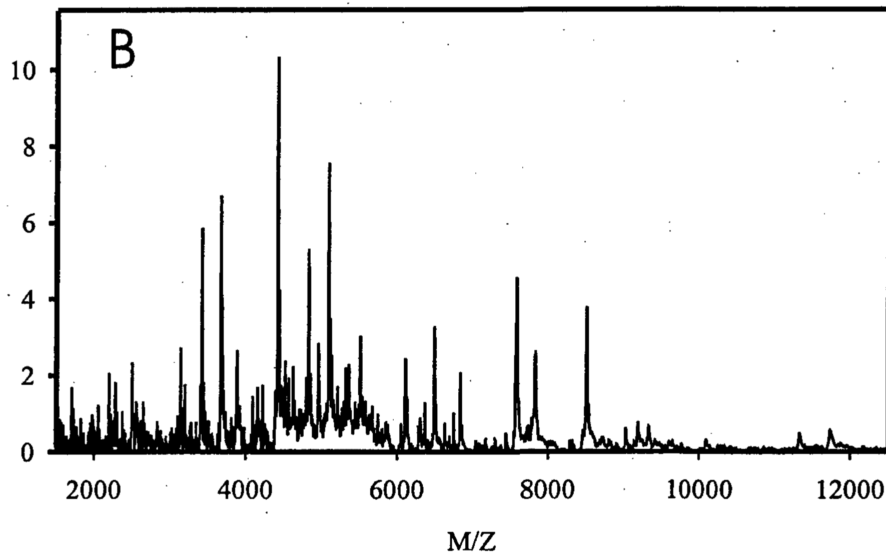


Figure 2. SELDI-TOF MS analysis of eluted protein. Protein from fraction 5 were analyzed directly (Panel A) or following exhaustive trypsin digestion (Panel B). The proteins was spotted onto a GOLD proteinCHIP and the EAM used was SPA.

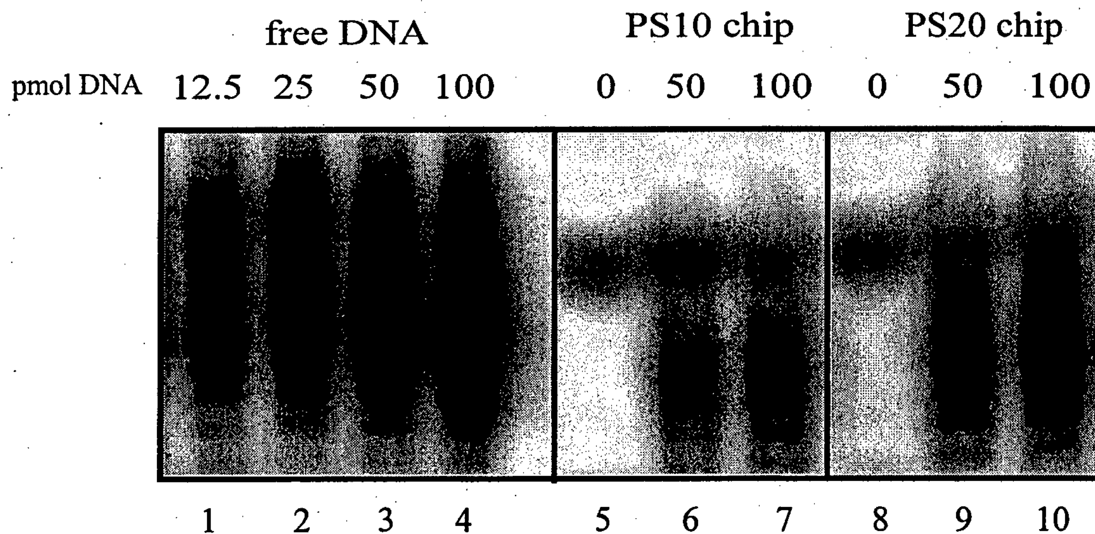


Figure 3. Terminal transferase labeling of DNA to determine binding efficiency. The concentrations of DNA indicated in the figure were either directly labeled with [a-P32]dGTP and terminal transferase (lanes 1-4) or following binding to the Ps10 (lanes 5-7) or Ps20 (lanes8-10 proteinCHIP. The signal in lanes 5-10 represent the DNA not bound to the CHIP following derivitization with the indicated amount of DNA.