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14. ABSTRACT

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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Table of Contents

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

Completion of Specific aims

Our original SOW presented two specific aims. Our progress toward completion of Aim 1 was presented in last years report and was reproduced in our second annual report as we indicated in the report. However, upon review this was deemed unnecessary and therefore we have removed that information which was included in the first annual report. Our progress towards completion of Specific Aim 2 is presented below and represents the majority of our effort this past year. We have applied for and received a 1 year no-cost extension to complete the studies with remaining funds. I have restricted this report to cover the progress that was made during the second years of funding and have responded to the comments made by the reviewer of our second annual report.

Progress during year two

Aim 1. Develop methodologies to allow the analysis of proteins with an affinity for cisplatin-damaged DNA to be identified via proteomics technology.

Aim 2. Determine the cisplatin-damaged DNA binding protein expression profiles for a series of ovarian cancer cell lines with documented sensitivity or resistance to cisplatin.

During year 2 of funding we have made considerable progress towards completion of the aims of our proposal. I have broken down our progress by specific aim. Towards completion of aim 1, we have continued to refine and optimize the methodologies to allow proteomic analysis of DNA repair proteins via SELDI-TOF MS analysis.

Our procedure involves derivatizing a chemically modified MS target with a modified DNA and performing fractionation and analysis directly on the SELDI target. In our annual report for year 1, we presented data on the efficiency of derivatization of the SELDI CHIPS. Having DNA bound to the chip we the asked if the DNA able to be bound by cellular proteins. We therefore applied known DNA repair proteins, RPA (Figure 1) and Ku (Figure 2), to the DNA modified chips. Unbound protein was removed by three washed and the bound protein directly analyzed after the addition of EAM. The results shown in Figure 1 demonstrate the ability to retain these proteins of the derivatized proteinCHIP arrays. Importantly, BSA was included with the RPA and Ku

and the inability to detect BSA demonstrates the specificity. Control experiments demonstrated that un-derivatized chips gave significantly reduced signal and heat denaturation of RPA abrogated binding.

The detection of these large proteins even in purified samples was very good though calculation revealed that a relatively small percentage of the applied protein was in fact retained by the modified DNA matrix. We also were able to observe the p70 subunit of RPA though the detection efficiency was considerable reduced compared to the p14, p34 and XPA proteins.

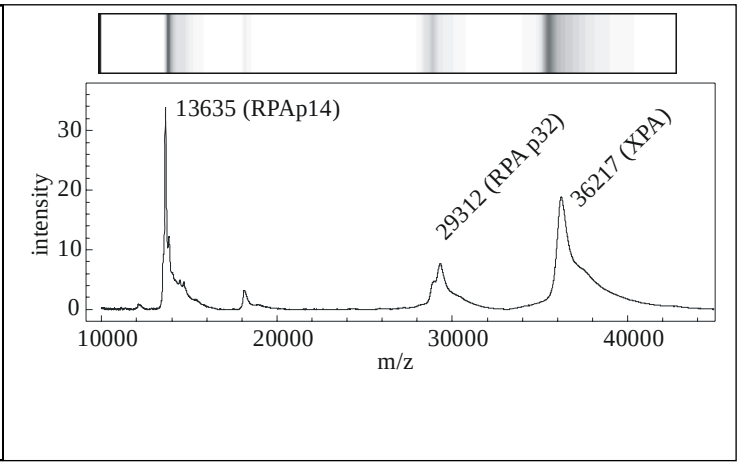


Figure 1. Retention of RPA on a DNA derivatized ProteinChip array. Purified RPA was applied to the ProteinChip and allowed to bind for 60 min at 25°C. The unbound protein was washed from the spot and allowed to dry. EAM was applied and the chip analysed in the PBSII SELDI-TOF MS. The top panel is an SDS gel representation of the spectrum obtained on the bottom panel.

The analysis of the Ku protein binding to the DNA modified chip was event less efficient compared to RPA and XPA. The results presented in Figure 2 show a representative analysis. In this case while clearly detectable, the amount of Ku retained and detected was estimated to be 1% of the applied protein. The Ku 80 protein actually has a calculated mass of 86, consistent with the SELDI-TOF analysis. This relatively low level of signal is likely the results of the inefficiency of the desorption and ionization of the large proteins molecules.

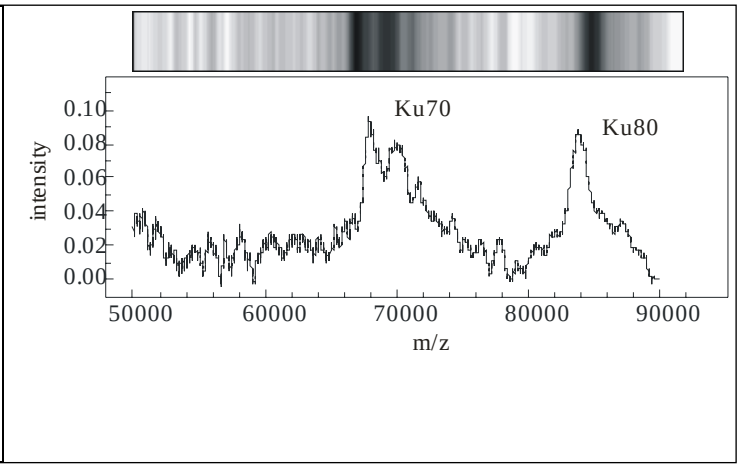


Figure 2. Retention of Ku on a DNA derivatized ProteinChip array. Purified Ku was applied to the ProteinChip and allowed to bind for 60 min at 25°C. The unbound protein was washed from the spot and allowed to dry. EAM was applied and the chip analysed in the PBSII SELDI-TOF MS. The top panel is an SDS gel representation of the spectrum obtained on the bottom panel.

Therefore we pursued on chip tryptic digestion of the retained proteins as described on our initial application. This proved to increase the sensitivity of the detection of each protein in addition to allowing the fingerprint of each protein to be determined. A representative analysis is presented in figure 3. In these experiments the proteins were bound to the DNA derivatized chip and unbound proteins removed by washing. The bound protein was then treated with trypsin at various concentrations on

the chip and digestion performed for a range of time. The digestion reactions were terminated by the addition of EAM. The chips were placed in a humidified chamber to reduce evaporation of the small volumes used in these analyses. The increased sensitivity observed in the RPA Panel A, XPA panel B and the combination of the two proteins on a single spot Panel C is evident by the y-axis intensity values. Comparison of the spectra with that of purified RPA or XPA treated with trypsin in solution and them applied to the chip revealed a coincidence of peaks present in each spectra.

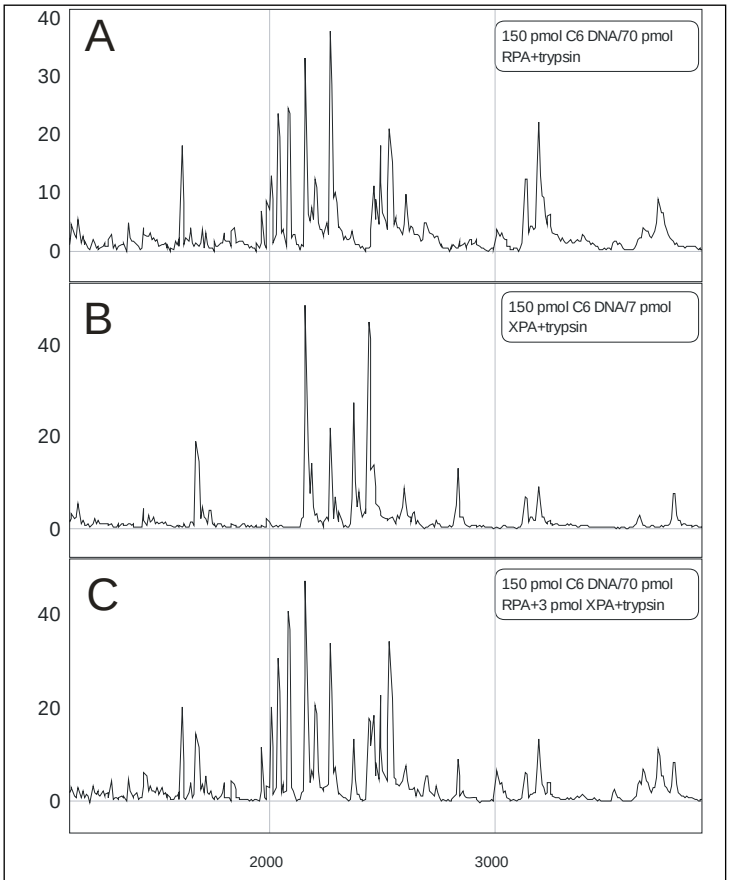


Figure 3. On-chip tryptic digestion of proteins retained on a DNA modified CHIP.

The next step with the application of cell extracts to the DNA derivatized chips. This step proved more difficult in that the sensitivity of the intact, undigested proteins was limited, as we demonstrated in the year one progress report where we were able to detect specific undigested proteins in the low molecular weight range (<15kDa). We pursued the analysis of these extracts and retained proteins on the chip by tryptic digestion, which again increased sensitivity, but also dramatically increased the complexity of the analysis. While peak identification analysis and computer searches where pursued we also undertook the definitive identification of the proteins retained on the DNA modified chip. We fractionated the extracts on DNA modified beads similar to the DNA modified SELDI CHIP and analyzed the retained proteins. The first methodology involved direct tryptic digestions of the protein pool and MALDI-TOF analysis. SELDI on the DNA modified CHIP was not used as the proteins were digested with trypsin and therefore have lost biologic activity. A representative analysis is presented in figure 4. The full MALDI-TOF spectrum is presented in the center panel and expanded in the peripheral sections. Clearly excellent signal intensity and resolution was observed.

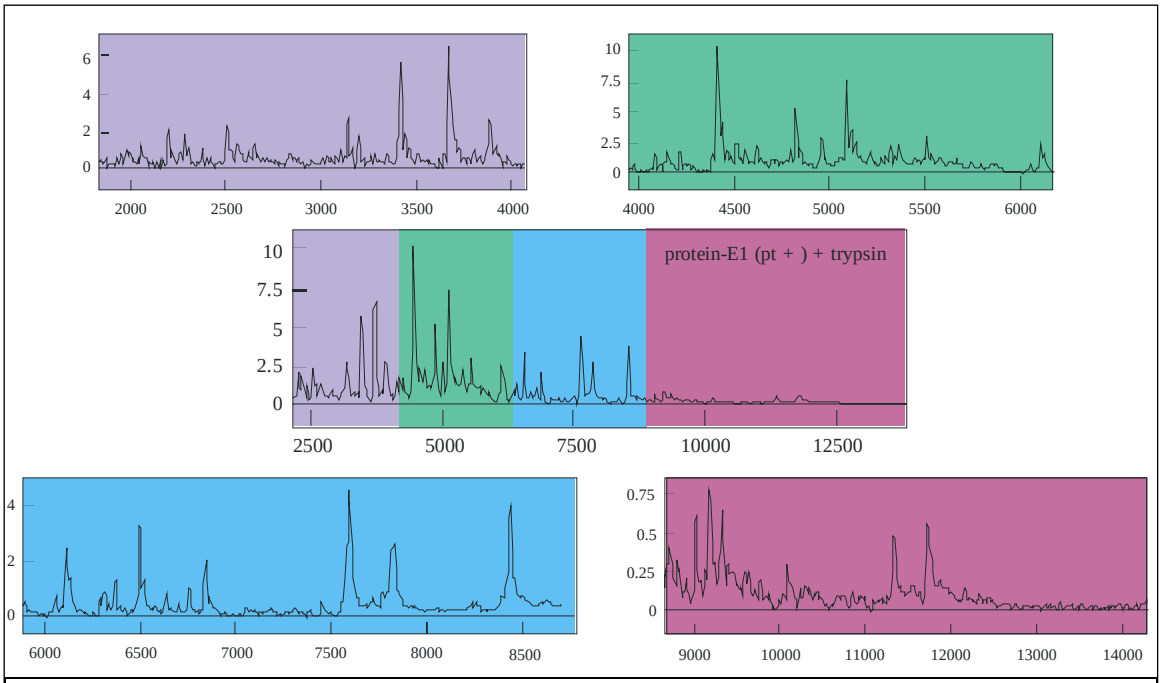
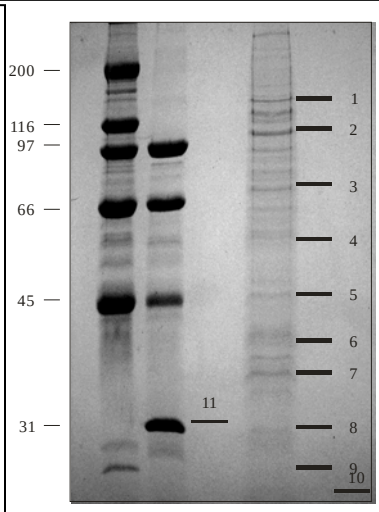


Figure 4. MALDI-TOF MS analysis of tryptic fragments from E1 fraction. MALDI-TOF MS analysis was performed as described earlier. Protein from the E1 fraction was digested with trypsin by incubation overnight at 37 C and peptide fragments analyzed. The center panel represents the entire spectrum collected and the outlying panels are expanded views of the indicated regions. The x-axis is M/Z and y-axis is the signal intensity.

Based on these results we then set out to identify the individual proteins. This was accomplished by SDS-PAGE separation and in-gel tryptic digestion of individual proteins. A representative example of an SDS gel is presented in Figure 5. Following digestions the peptides were analyzed by MALDI-TOF MS. A representative spectra is presented in Figure 6. The top panel represents the trypsin control and the middle panel digestion of the band from position 1 on the gel in Figure 5.

Figure 5. Identification of individual cisplatin-DNA binding proteins. SDS-PAGE analysis of fraction E1 detected by colloidal blue staining. The bands corresponding to each numbered line were excised. Controls bands 10 and 11 represent a blank lane and the 32kDa carbonic anhydrase protein, respectively. The gel slices were processed for in-gel trypsin digestions as described earlier.



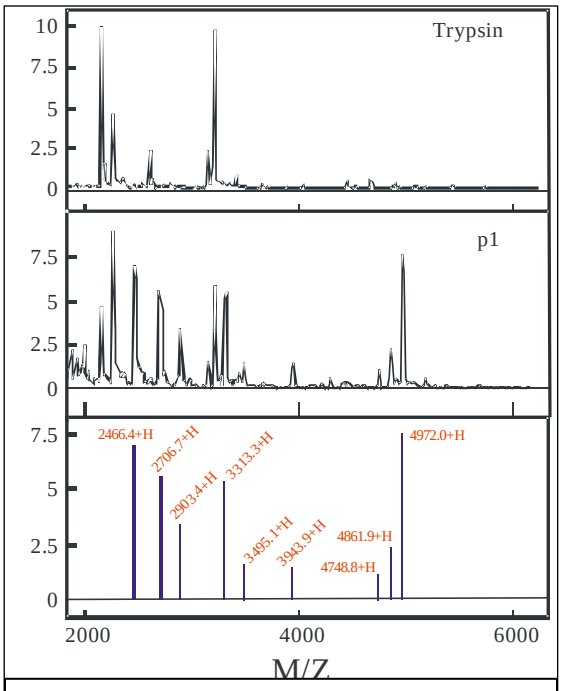


Figure 6. The top panel is the MS analysis of band 10 and represents a trypsin control. The middle panel in the spectrum from band 1 and the bottom panel represents analysis of the peaks specific to band 1 subtracting those present in both spectrum.

Each protein was processed independently and MASCOT searched performed to identify the individual proteins. The list to date is presented in Tables 1 and 2.

Band	# peaks	min. match	tolerance (Da)	mw range (kDa)
1	9	6	4	135-165
1	20	11	4	135-165
3	19	9	4.5	66-80
4	11	5	4.5	49-61
8	19	7	4.5	26-32
11	23	9	2.5/4.5	24-36

Table 1.

BAND	# matches	ID	Description
1	12	DPG1_HUMAN	DNA polymerase gamma
	12	MSH6_HUMAN	MutS alpha 160 kDa subunit
	12	FACA_HUMNA	Fancon anemia group A protein
3	4	FXR2_HUMAN	Fragile X syndrome related protein 2
4	4	MDM4_HUMAN	MDM4 p53 binidng protein
8	6	RA51_HUMAN	Rad51 splice isoform 2
	7	FKB7_HUMAN	FK506 binding protein 7
11	9	CAH2_Bovine	Bovine carbonic anhydrase

Table 2.

In addition we have employed a modification of the procedures that allows higher throughput and employs biotin modified DNA and a strepavidin matrix. The proteins identified in this manner have confirmed our original analyses and is being pursued for the other cell lines. These analyses are being continued to validate the initial spectra and will be completed in the coming year using existing funds in the no-cost extension.

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.

- Established protocol for biotin-streptavidin isolation of DNA damage recognition proteins and their analysis of MALDI-TOF MS.

- Identified the mcm2 protein in the selected pool of DNA damage proteins and confirmed it’s selection by western blot analysis.

Reportable outcomes

1. Jason A. Lehman and J**ohn J. Turchi.** MALDI-TOF Mass Spectrometry Analysis of the Human Ku Heterodimeric Protein for DNA-Binding Regions**.** 6th Annual Midwest DNA repair, Lexington KY, June 2003

2. Jiazhen Wang and **John J. Turchi.** Analysis of DNA Repair Protein Expression in Cisplatin-Resistant Ovarian Cancers by SELDI-TOF Mass Spectrometry. 6th Annual Midwest DNA repair, Lexington KY, June 2003

Conclusion

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.

References

Boente,M.P., Hamilton,T.C., Godwin,A.K., Buetow,K., Kohler,M.F., Hogan,W.M., Berchuck,A., and Young,R.C. (1996). Early ovarian cancer: a review of its genetic and biologic factors, detection, and treatment. Curr. Probl. Cancer *20*, 83-137.

McGuire,W.P. and Ozols,R.F. (1998). Chemotherapy of advanced ovarian cancer. Semin. Oncol. *25*, 340-348.

Mills,G.B., Bast,R.C.J., and Srivastava,S. (2001). Future for ovarian cancer screening: novel markers from emerging technologies of transcriptional profiling and proteomics. J. Natl. Cancer Inst. *93*, 1437-1439.

Perez,R.P., Godwin,A.K., Hamilton,T.C., and Ozols,R.F. (1991). Ovarian cancer biology. Semin. Oncol. *18* , 186-204.

Srinivas,P.R., Srivastava,S., Hanash,S., and Wright,G.L.J. (2001). Proteomics in early detection of cancer. Clin. Chem. *47*, 1901-1911.