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ABSTRACT

The successful treatment of prostate cancer requires detection of the disease at early stages. Currently the early diagnosis of prostate cancer largely depends on the detection of prostate-specific antigen (PSA) in circulation. However, PSA can only precisely detect 40% of prostate cancer and is not specific for the occurrence of prostate cancer. We reasoned that the success and accuracy in early diagnosis of prostate cancer may be significantly improved if a panel of prostate cancer-specific markers can be identified and used in combination for detecting early stage of prostate cancer. In the first year of the funding period, we constructed cDNA library in our pTRAP1 retroviral plasmid using RNA isolated from human prostate tumor samples. In this second year of funding period, we focused our studies on generating human prostate tumor cDNA library in which the signal peptides are enriched. In the third year of this funding we will identify both secreted and cell surface proteins overexpressed in early stage prostate tumors.

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Introduction

The successful treatment of prostate cancer requires detection of the disease at early stages. Currently the early diagnosis of prostate cancer largely depends on the detection of prostate-specific antigen (PSA) in circulation. However, PSA can only precisely detect 40% of prostate cancer and is not specific for the occurrence of prostate cancer. We reasoned that the success and accuracy in early diagnosis of prostate cancer may be significantly improved if a panel of prostate cancer-specific markers can be identified and used in combination for detecting early stage of prostate cancer. Our proposal aims to identify a panel of secretion proteins overproduced in early stage prostate tumors. We believe that some of these proteins can be potential candidate biomarkers for the early diagnosis of prostate cancer. Through subsequent studies that are beyond the scope of this proposal, these proteins can be further analyzed for their value as early diagnostic markers for breast cancer.

Body

The goals of our research in the second year of this funding are to 1) create a secreted and cell-surface protein library (month 14-21); and 2) array the library onto DNA chips (month 22-24). In our first 14 month work of this funding, we constructed a secretion trap library with 1.5×10^7 independent clones from 23 human prostate tumor samples. During month 14-21, we performed two secretion trap screens with the constructed library in order to isolate cDNA fragments encoding the signal sequences of the both secreted and cell-surface proteins in human prostate tumor tissues. The detailed two screens are described in the following.

I. Screen #1.

In first screen, we packaged the library as a whole into retroviruses by transfecting the library DNA into a retroviral packaging cell line, LinX-A. A total of 100 10-cm dishes of 293 cells (2×10^6 cells/dish) were infected with the library viruses, and a parallel infection with a control virus containing LacZ gene indicated an infection efficiency of over 30%. We thus estimated that at least 5×10^7 cells had been infected, which was sufficient to cover the complexity of the library with a more than 2-fold redundancy assuming 2×10^7 cDNA in the library. After 1-week hygromycin selection, infected cells were incubated with magnetic beads coupled to an anti-CD8 antibody to isolate cells expressing cell surface CD8. Because the yield of the first round of selection was expected to be low due to the low percentage of CD8 positive cells in the initial population, we combined all isolated cells in two large pools in order to shorten the period of recovery before the cells were ready for the second round. Cells were detached from each plate with 10mM EDTA and combined into 2 pools, with each pool containing 5×10^8 cells from 50 plates. Each pool was incubated with 2.5 ml of Dynabead M-450 CD8 in 50 ml of PBS containing 5% fetal calf serum and 0.6% sodium citrate in 50 ml Falcon tubes. After washing with the same buffer, the bound cells were eluted with 500 μ l of DETACHaBEAD, and plated into four 10-cm plates. The subsequent rounds of selection was performed in Eppendorf tubes with 50 μ l of Dynabead M-450 CD8 in a total volume of 1 ml, and the bound cells were eluted with 10 μ l of DETACHaBEAD. After each selection cycle, a portion of the recovered cells was immuno-stained with an anti-CD8 antibody coupled to a florescent dye (FITC), and the percentage of CD8 positive cells was determined by flow cytometry. After four rounds of selection with CD8 beads, the CD8 positive cells were enriched to about 80% for both pools.

To recover the integrated proviruses, genomic DNA was isolated from above enriched cells and then treated with Cre recombinase. The excised proviruses DNA were electroporated into *E. coli*, and 20 single colonies were first analyzed by DNA sequencing to determine the identity of the cDNA fragment in each clone. Unfortunately, we found that both pools were dominated by 2-3 cDNA species. This finding indicated that this screen failed due to the overgrowth of a few cells that had acquired growth

advantage. Thus, the lesson we have learned from our first screen is that cells transduced with the library need to be kept as separate as possible during selection and growth.

II. Screen #2.

The strategy for our second secretion trap screen was designed to keep the cells containing the library in as many pools as possible during the selection process. The glycerol stock of the library was divided into 96 pools, each containing 2×10^5 bacterial cells. Plasmids were isolated from these pools using a Qiagen robot. These pools of DNA were transfected separately into LinX-A packaging cells, and the resulting virus pools were used to infect 293 cells in 96 10-cm plates (2×10^6 cells/dish). At an infection efficiency of 30% (see above), we estimated that each pool of the library had been transduced into 4×10^5 cells, and that the total number of cells transduced by the library was approximately 4×10^7 . After hygromycin selection, 10^7 of cells from each pool were selected separately with 50 μ l of anti-CD8 beads in a total volume of 1 ml in a microfuge tube, and the bound cells were eluted with 10 μ l of DETACHaBEAD. Eluted cells from each set of 4 plates were combined and plated into a fresh 10-cm plate for recovery. After the first round of selection, the number of pools was reduced to 21, which were named A to U. These pools were kept separate during the following rounds of selection, which again was performed in microfuge tubes with 50 μ l of Dynabead M-450 CD8 in a total volume of 1 ml.

After each round of selection, a portion of the recovered cells was stained with an

POOL L#	Pre- Selection	First Round	Second Round	Third Round	Fourth Round
A	0.2	1.8	5.2	24.1	57.3
B	0.1	1.2	2	20	60.7
C	0.1	1.5	6.7	43.3	68
D	0.1	0.9	1.1	12.3	47.5
E	0.1	1.1	2.9	21.9	68.6
F	0.2	2.6	16.7	73.8	87.2
G	0.6	2.5	10.4	44.3	75.6
H	0.1	0.6	6.8	44.3	70.2
I	0.2	1	10.2	43.3	68.6
J	0.2	2.7	12.3	44.6	63.5
K	0.2	0.9	1.5	21	49.2
L	0.1	0.8	1.4	13.5	49.8
M	0.2	2	9	48.3	62.5
N	0.2	0.8	7.2	34.3	61.8
O	0.2	1.2	4.6	19.1	40.1
P	0.1	0.7	6.1	23.1	49
Q	0.1	0.5	5.5	34	48.4
R	0.1	1.1	14.5	27.5	44.2
S	0	0.6	4.3	21.7	49.5
T	0.3	3.8	40.1	66.2	67.1
U	0.2	0.9	13.2	40.3	63.7

Table 1. % of CD8 positive cells after each round of selection with magnetic beads coupled to an anti-CD8 antibody. The initial cell populations were transduced with pools of the secretion trap library from human breast tumors. After each round of selection, a portion of the cells was stained with an anti-CD8 antibody coupled to FITC and analyzed by flow cytometry.

anti-CD8 antibody coupled to FITC, and the percentage of CD8 positive cells were determined by flow cytometry. After 4 rounds of selection, all pools contained more than 40% of CD8 positive cells (Table 1). As controls, we also performed parallel selection with 293 cells transduced with either pTRAP alone or a combination of

pTRAP containing an EGF signal sequence (pTRAP-SS) and pTRAP mixed at a ratio of 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} or 10^{-6} . As expected, the CD8 positive cells were gradually enriched in the

control cell populations transduced even with the lowest percentage (10^{-6}) of pTRAP-SS, but not in that transduced with the pTRAP vector only (Table 2). These results suggest that the selection was successful.

	Pre- Selection	First Round	Second Round	Third Round	Fourth Round
0	0.1	0.4	0.3	0.4	0.5
10^{-2}	10.6	46.1	72.4	83.6	94.5
10^{-3}	0.3	14.1	45.4	76.5	81.5
10^{-4}	1	21.9	33.4	55.4	70.2
10^{-5}	0.4	1.4	26.5	43.2	62.8
10^{-6}	0.1	0.3	11.4	39.2	45.8

Table 2. % of CD8 positive cells after each round of selection with magnetic beads coupled to an anti-CD8 antibody. The initial cell populations were transduced with a mixture of the pTRAP vector containing an EGF signal sequence and the pTRAP vector mixed at a ratio of 0, 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} . After selection, part of the cells was stained with an anti-CD8 antibody coupled to FITC and analyzed by flow cytometry

The proviruses were recovered from these pools in which CD8 positive cells had been enriched to more than 40%. Restriction analysis of randomly selected clones from each pool indicated that most of these pools were represented by multiple cDNA species of different sizes, with the exception of 2 pools that seemed to be dominated by 2-3 different types of cDNA. Further DNA sequence analyses on the randomly selected clones from the remaining 19 pools also indicate that these pools contain different cDNAs. Therefore, it seems that our new strategy has solved the problem we encountered in our first screen, and that the domination of a small number of clones did not occur in the majority of the pools in the second screen (19 out of 21 pools).

Due to the unexpected failure on our first screen, we are currently in the process of normalizing the generated library and expected to finish this part of work in a few weeks. This is about 1 month behind what we previously scheduled. Once we finish the normalization, we will start micro-array analysis.

Key Research Accomplishments

We have obtained a human prostate tumor library in which the signal sequences are enriched.

Reportable Outcomes

We have obtained a signal peptide-enriched human prostate tumor library.

Conclusions

We have obtained a human prostate tumor library in which the signal sequences are enriched. The micro-array analysis will be carried out after further validation of this library by DNA sequencing.

References

None

Appendices

N/A