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Award Number: W81XWH-08-1-0593

TITLE: Induction of huntington protein as a novel strategy for prevention and treatment of breast cancer

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REPORT DATE: March 2010

TYPE OF REPORT: final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-03-2010		2. REPORT TYPE Final report		3. DATES COVERED (From - To) 1 SEP 2008 - 28 FEB 2010	
4. TITLE AND SUBTITLE Induction of huntingtin protein as a novel strategy for prevention and treatment of breast cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0593	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jin-Rong Zhou, Hamid Abdolmaleky				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Beth Israel Deaconess Medical Center Boston, MA 02215				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Material Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The objective of this study was to determine the role of the Huntingtin network genes in the progression of breast cancer. HAP1, HIP1 and HTT expression, DNA methylation status and histone acetylation of HAP1, HIP1 and HTT promoters were determined. HAP1 gene was silenced in breast cancer cell lines and in much lower extent in immortalized MCF10A cells, but not in normal human mammary epithelial cell line (HMEC). HAP1 expression was also dramatically reduced in breast tumors and adjacent normal breast tissues, compared with normal breast tissues of healthy women. Epigenetic analysis showed that HAP1, but not HTT or HIP1, had promoter DNA hypermethylation in breast cancer cell lines versus HMEC, and in breast cancer and adjacent normal breast tissues of patients versus normal controls. Further experiments using HTT and HAP-1 expressing plasmids <i>in vitro</i> showed that cell growth rate is significantly reduced in cancer cell lines. The results support that HTT and especially HAP1 may play functional roles in breast cancer development and progression. More importantly, HAP1 may have a protective role against breast cells from neoplastic transformation.					
15. SUBJECT TERMS huntingtin protein, breast cancer, gene function assay					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Despite extensive progress in basic and translational research and clinical therapy, breast cancer (BRCA) remains the most common cancer and the second leading cause of cancer related death among women in the US. It has been recognized that the more effective approach to combat BRCA is via early prevention by applying chemopreventive approaches. Significant prevention of BRCA can be achieved by applying preventive regimens to modulate the targeted molecule/signaling pathway that plays a significant role in the development and progression of BRCA. Thus, the searching for novel and functional molecular targets remains the top priority in BRCA research and becomes a prerequisite in designing effective and safe chemopreventive and therapeutic regimens against BRCA.

Epidemiological investigations have demonstrated that the overall cancer risk in Huntington's disease (HD) patients is significantly lower than that in their first-degree relatives or general population, suggesting that some biological factors in HD may have cancer prevention activity. Identification of those endogenous biological factors may not only lead to a better understanding of BRCA etiology and development, but also provide novel and functional molecular target(s) for designing preventive strategies against the development and progression of BRCA.

HD is an inherited neurodegenerative disorder caused by abnormal CAG repeat expansion in the first exon of the huntingtin (HTT) gene, resulting in an expanded polyglutamine tract in huntingtin protein. HTT interplays with a large numbers of proteins and participates in diverse cellular pathways. Several HTT binding partners in the network have been identified, which include the HTT-Interacting protein-1 (HIP1) and HTT-associated protein-1 (HAP1). Although huntingtin related components (HTT, HAP1 and HIP1) were originally identified in neuronal cells, they were subsequently found in other cells and organs including mammary epithelial cells. However, their roles in BRCA have not been investigated.

BODY

Our hypotheses in this research topic are that increased expression of HTT in normal mammary epithelial cells may have a protective effect against the development of BRCA, and induction of HTT in cancerous breast cells may reverse/improve the phenotypic characters of breast cancer cells. The objective of this concept award application is to generate critically important experimental evidence to support the hypothesis. Specific aim 1 is to compare the expression levels of HTT in normal and cancerous breast cells. Specific aim 2 is to determine the effects of HTT manipulation on cellular behaviors of normal breast epithelial cells and BRCA cell lines in vitro. Specific aim 3 is to determine if increased HTT expression in BRCA cell lines will alter tumor growth in vivo. The experimental findings in these specific aims are shown as follows.

Experimental findings in specific aim 1

Altered gene and protein levels of Htt, HAP1 and HIP1 in normal and cancerous breast cells: We first determined the expression of HTT, HIP1 and HAP1 in normal and cancerous breast cells in cell lines and in tissues. We found that HTT expression was generally downregulated (Fig 1A) and HIP1 expression was upregulated (Fig 1B, except MB453) in breast cancer cell lines, compared with normal breast epithelial cells (HMEC). Whereas these differences were not observed in clinical normal breast tissues vs breast tumors (Fig 1D, 1E).

On the other hand, HAP1 gene expression was silenced in BRCA cell lines (Fig 1C) and breast

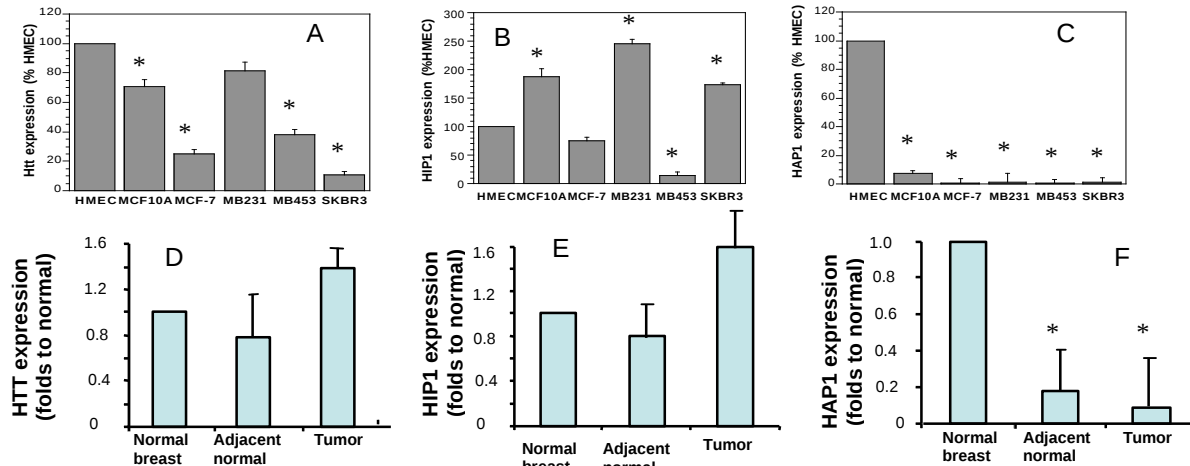


Figure 1. Expression of HTT, HIP1 and HAP1 genes in normal vs cancerous cultured cells and in human tissues. Estrogen-dependent (MCF-7 and MDA-MB453) and estrogen-independent (MDA-MB231 and SKBR3) human breast cancer cell lines, immortal mammary cell line MCF-10A and normal human mammary epithelial cell (HMEC) were used to determine the differential expression of HTT (A), HIP1 (B) and HAP1 (C). Normal breast tissues from healthy women, breast tumors and adjacent normal breast tissues from BRCA patients (n=10/group) were also used to determine the differential expression of HTT (D), HIP1 (E) and HAP1 (F).

tumors (Fig 1F), compared with that in normal mammary epithelial cells (HMEC) and normal breast tissues of healthy women, respectively. More strikingly, HAP1 gene expression was also dramatically reduced (80-90%) in immortalized MCF10A cells and in adjacent normal breast tissues of BRCA patients, when compared with that in HMEC and normal breast tissues of healthy women, respectively. But no significant correlation of HTT or HIP1 expression with BRCA was found. This robust finding that HAP1 expression was largely silenced in adjacent breast tissues of BRCA patients and in non-cancerous immortal MCF-10A cells provides strong supporting evidence to suggest that HAP1 may play a significant role in the early event of BRCA development. Specifically, it suggests that HAP1 may have a protective role against mammary epithelial transformation, and loss of HAP1 function may be an important early causal factor for BRCA development.

Promoters DNA methylation of Htt, HAP1 and HIP1 genes in breast cancer cells:

To determine if altered expression of genes is modulated by DNA methylation, we further determined the DNA methylation status of HTT related genes. The MSP analysis was used to screen and to assess the methylation status of the selected group of specific CpGs in the genes promoter region confirmed by subsequent bisulfite sequencing, and immuno precipitation of methylated DNA using methyl collector kit. Both of the methods showed that Htt and HIP1 promoters' DNA are totally unmethylated in normal as well as cancer cell lines (Fig 2A). However, HAP1 promoter region showed partial DNA methylation at several CpG sites including one flanking SP1 binding site in cancer cells (Fig 2A). QMSP analysis also confirmed increased HAP1 methylation in BRCA cell lines (Fig 2B) and in breast tumors (Fig 2C) compared with the controls. These results suggest that one of the mechanisms that HAP1 is silenced in breast cancer cells is due to DNA promoter hypermethylation.

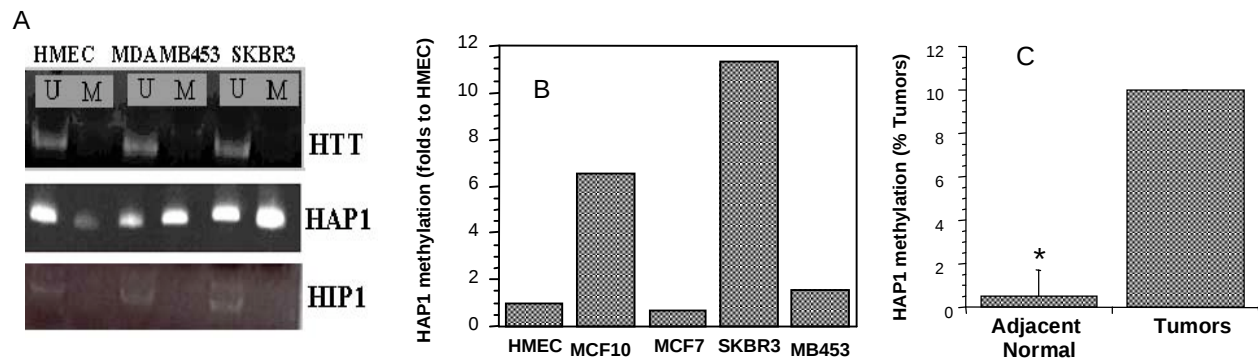


Figure 2. DNA promoter hypermethylation of HAP1 gene in breast cancer cells and breast tumors. A: MSP analysis showing high degree of promoter DNA methylation of HAP1 gene and un-methylation (or very low methylation) of HTT and HIP1 promoters in M B453 and SKBR3 versus normal human mammary epithelial cells. U: unmethylated, M: methylated PCR product. B: QMSP analysis showing increased DNA methylation in HAP1 gene promoter in breast cancer cells, compared with HMEC. C: QMSP analysis showing increased DNA methylation in HAP1 gene promoter in breast tumors, compared with paired adjacent normal breast tissues (n=4 pairs). The Value with “*” is significantly different from the control.

Histone acetylation of Htt, HAP1 and HIP1 in cell lines: To further determine if altered gene expression is in part due to histone modification we analyzed histone-3 acetylation level using CHIP and quantitative real-time PCR analysis of precipitated DNA in HMEC and MCF7 cells as described under method. Unexpectedly, chromatin acetylation of Htt, HAP1 and HIP1 was almost 3-4 times more in MCF7 compared to HMEC cells suggesting that histone acetylation might be a compensatory mechanism to restore the expression of these genes in cancer cells. Instead of MCF7 chromatin acetylation of Htt, HAP1 and HIP1 in MB231 was similar to HMEC. Breast cancer cells were also treated with 5-azaC (a DNA demethylating agent) and sodium butyrate (a HDAC inhibitor). We found that 5-azaC treatment significantly increased HAP1 expression by over 100 times, but sodium butyrate had little effects.

The results derived from the experiments at aim 1 HTT and HAP1, especially HAP1, may be important candidate biomarkers related to breast cancer. Since the results showed downregulation of HTT and especially HAP1 in breast cancer cells, our further investigations were focused on these genes.

Experimental findings in specific aim 2

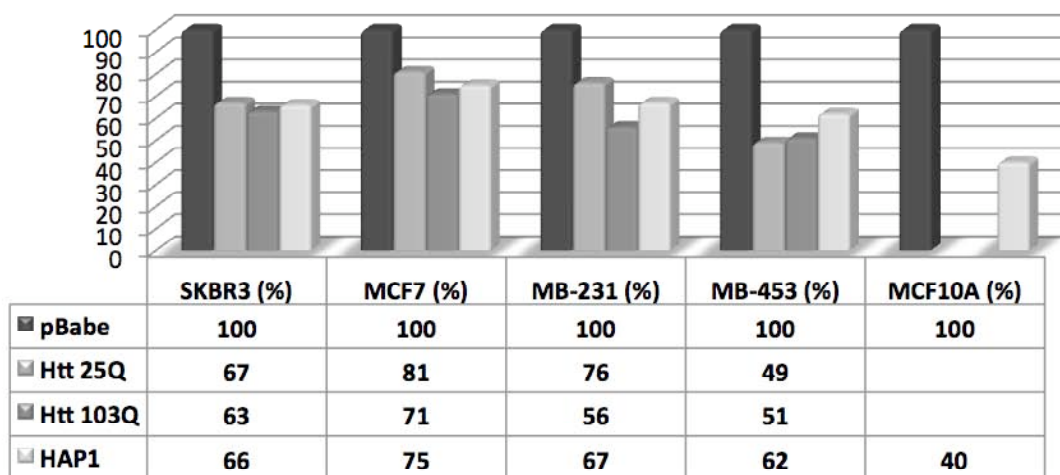
Effects of overexpression of genes on cell growth: In order to investigate the effects of HTT and HAP1 on cell growth, plasmids were synthesized to express HAP1 and HTT (25Q; normal repeat size and 103Q; expanded repeat). Successful transfection of HTT was confirmed by RT-PCR. For the analysis of HAP1 expression in HAP1-puro stable cell line we used qRT-PCR that showed >1000-folds increase in HAP1 expression compared to cells transfected with pBABE-puro alone.

Cell growth assay showed that hyperexpression of HTT or HAP1 was associated with a significant decrease of growth rates (~25-40%) in breast cancer cell lines (Figure 7) in general, but with a more significant decrease of the growth of MCF10A cells by over 60% (HAP1-overexpressing cells), compared with that of the vector-controls. These results not only support the effect of HAP1/HTT overexpression on reducing breast cancer cell growth, but also suggest that restoration of HAP1 function in the early stage of BRCA development (non-neoplastic

lesion) may have a more significant impact on BRCA prevention than that in the later stages of BRCA.

In MB-453 cells we analyzed the cells growth rate at 3, 5 and 7 days time point and found that the effects of HAP1 and HTT-103Q transfection on the inhibition of cell growth were progressive over time. We also found evidence that the transfected cells eventually died over weeks and cells living in the culture were those cells that lost HTT or HAP1 expression or were not transfected with HTT or HAP1 from the beginning. Note that, since a minority of pBABE plasmid following enzymatic restriction may not catch the insert as the efficiency of the enzymatic restriction and/or ligation is not 100% during plasmid synthesis, some empty vectors may transfect the competent cells (E-coli) at the same time. As the result, the selected colony may contain empty pBABE along with the vector containing the insert. These empty vectors are mixed with the purified plasmid carrying the insert and may transfect a minority of cells in culture donating puromycin resistance to them. These cells dominate the cell culture plate over time upon the death of cells expressing high level of HTT or HAP1. In order to confirm the loss of HAP1 or HTT expression over time we used qRT-PCR and analyzed gene expression level in cultured cells in successive weeks and found decrement of HAP1 expression in each week (~2 Ct value versus β -Actin in each week).

Figure 7. Cell growth assay in various stable cell lines expressing HAP1 and Htt vs. control cells (pBabe-Puro)



Effects of knockdown of genes on cell growth: To determine the effect of HAP1 knockdown on the cellular behaviors of non-neoplastic cells (MCF10A and HMEC), we first conducted studies aiming to establish MCF10A/HAP1-shRNA stable cell line by using PLKO.1 cloning vector (Addgene). The forward and reversed oligos (HAP1 shRNA) annealed and inserted in PLKO were cut by Age1 and EcoR1 using standard T4 ligation. The plasmids were transformed to bacteria (competent cell) and cultured in LB agar containing 100 μ g/ml ampicillin. Selected colonies were cultured again in LB and purified plasmid was sent for sequencing to confirm the presence of insert. MCF10A cells were transfected with plasmid using fusion according to the manufacturer instruction. After two days transfected cell were selected by

puromycin treatment to generate the stable cell lines. HAP1 expression decreased by 75% in these cells, compared with the cells transfected with PLKO-puro empty plasmid.

We further tried to transfect HAP1-shRNA into HMEC cells. Although we were unsuccessful to generate stable cell line, we were successful to increase cell viability up to six weeks. Of note that, upon puromycin selection ~99% of cells died indicating that the efficiency of transfection was low, as expected in normal cell lines. Meanwhile, in this attempt we observed higher cell growth rate (almost double) in transfected HMEC cells versus untransfected cells. These results also suggest that knockdown of HAP1 may extend cell growth.

Experimental findings in specific aim 3

To further evaluate the functional roles of HTT and Hap1 in breast cancer growth and progression, we established the animal models of orthotopic breast tumors by injecting cells into mammary fat pads of immune deficient mice to determine the rates of tumorigenesis of breast cancer cells with different levels of HTT or HAP1. We evaluated the vector control, HTT-25Q-overexpressing, HTT-103Q-overexpressing and HAP1-overexpressing stable cell lines from SKBR3, and MB231 cell lines because the sublines showed higher efficiencies of overexpression. To our surprising, the tumor models were not effectively developed, and most mice did not develop tumors. For MB231 cells, HAP1-overexpressing cells showed more tumor development than the vector-control cells. We are determining the HAP1 status in these tumors to see if it is possible that the developed tumors have not been effectively transfected with HAP1. We are also trying to evaluate other cell lines (MCF7 and MB453) for tumor development.

KEY RESEARCH ACCOMPLISHMENTS

The following results were obtained from the experiments:

- (1) The in vitro studies demonstrated reduced levels of HTT and HAP1 gene expression and an increased level of HIP1 gene expression in breast cancer cell lines, compared with the normal mammary epithelial cells (HMEC). More importantly, the HAP1 gene expression in immortal MCF-10A cells was also dramatically reduced compared with HMEC;
- (2) The results derived from clinical tissues did not show significant alterations of HTT or HIP1 gene expression between normal and cancerous breast tissues, whereas HAP1 gene expression was dramatically reduced in both breast tumors and adjacent normal breast tissues of the patients, and no difference of HAP1 expression was found between breast tumors and adjacent normal breast tissues;
- (3) The above striking findings strongly support the hypothesis that HAP1 may be a more important molecular candidate than HTT that participates in the early stages of breast carcinogenesis process;
- (4) The gain-of-function assay showed that restoration of HAP1 and HTT reduced the growth of breast cancer cells, supporting that restoration of HAP1 and HTT may have therapeutic effect against the growth and progression of breast cancer;
- (5) The gain-of-function assay also showed that HAP1 overexpression had more potent effect on reducing the growth of MCF-10A cells than that of breast cancer cells, which suggests that targeting/manitaining HAP1 function in the early stages of

- mammary carcinogenesis may be more effective on breast cancer prevention than the late stages;
- (6) The loss-of-function assays showed extended cell growth/survival in normal cells, supporting the hypothesis that preventing HAP1 loss in the normal breast cells may have effective prevention activity against breast cancer development.
 - (7) The promising results derived from this concept award provide important supporting evidence to support further investigation to determine the preventive role of HAP1 and/or HTT in breast cancer development and progression.

REPORTABLE OUTCOMES

The results were presented at the following scientific meeting:

(1) S.H. Abdolmaleky, M.R. Eskandari, L. Li & J.-R. Zhou: Epigenetic modifications of huntingtin-related genes by dietary components for the prevention and treatment of breast cancer. Abst. # 394, AACR International Conference on Frontiers in Cancer Prevention Research, Houston, TX, December 6-9, 2009.

(1) J.R. Zhou, H. Abdolmaleky, M. Eskandari, G. Blackburn: Epigenetic modification of huntingtin associated protein-1 by bioactive natural components as a strategy for the prevention and treatment of breast cancer. Abst. #6533, Experimental biology'2010 annual meeting, Anaheim, CA, April 24-27, 2010.

The following manuscript has been submitted for publication consideration:

(1) M. Eskandari, H. Abdolmaleky, Y. Gong, L. Li, S. Thiagalingam, J.-R. Zhou: Epigenetic modification of huntingtin associated protein-1 in breast cancer development and progression. Submitted.

CONCLUSION

Our results support huntington disease related genes, HTT and especially HAP1 may play a functional role in breast cancer development and progression. More importantly, HAP1 may have a protective role against breast cells from neoplastic transformation. These robust results provide strong supporting evidence for proposing further research to determine the role of HAP1 in early stages of breast cancer development, and to define HAP1/HTT as functional targets for breast cancer prevention and treatment, and to develop novel breast cancer chemopreventive strategies by designing bioactive agents to target these functional molecules.

REFERENCES

None

APPENDICES

None

SUPPORTING DATA

None