Published OnlineFirst July 22, 2011; DOI: 10.1158/1055-9965.EPI-11-0379

Cancer Epidemiology, Biomarkers & Prevention

Research Article

Mitochondrial DNA Copy Number and Risk of Gastric Cancer: a Report from the Shanghai Women's Health Study

Linda M. Liao[1], Andrea Baccarelli[2], Xiao-Ou Shu[3], Yu-Tang Gao[4], Bu-Tian Ji[1], Gong Yang[3], Hong-Lan Li[4], Mirjam Hoxha[5], Laura Dioni[5], Nathaniel Rothman[1], Wei Zheng[3], and Wong-Ho Chow[1]

Abstract

Background: Mitochondrial DNA (mtDNA) is an approximately 16,000-bp circular double-stranded DNA molecule that is a prime target of oxidative damage. Several somatic mutations in mtDNA have been observed in gastric tumors, suggesting an involvement in gastric cancer risk and progression. mtDNA copy number in leukocyte DNA has also been linked to several other cancers, although the temporal relationship between mtDNA and cancer has not been adequately explored.

Methods: Using a nested case–control study design, we examined the association between mtDNA copy number in 162 gastric cancer cases and 299 matched controls within the Shanghai Women's Health Study, a large population-based prospective cohort. Relative mtDNA copy number was measured in triplicate by a quantitative real-time PCR assay in peripheral leukocytes.

Results: mtDNA copy number levels were comparable among cases and controls, with a median of 1.04 [interquartile range (IQR), 0.87–1.25] and 1.06 (IQR, 0.88–1.29), respectively. Overall, mtDNA was not associated with gastric cancer risk. However, the association differed when stratified by the time between sample collection and cancer diagnosis. An association between low levels of mtDNA copy number (<median) and gastric cancer risk was apparent among earlier diagnosed cases, in particular, those diagnosed within 2 years of sample collection (OR = 5.32; 95% CI = 1.03–27.60). This association was not present as the time between sample collection and cancer diagnosis increased.

Conclusions and Impact: Our findings suggest that there is no association between leukocyte mtDNA copy number and risk of developing gastric cancer; however, we observed a possible early disease effect on mtDNA copy number levels. *Cancer Epidemiol Biomarkers Prev; 20(9); 1944–9. ©2011 AACR.*

Introduction

Mitochondria are organelles found in all nucleated cells. The key role of mitochondria is to generate cellular ATP through oxidative phosphorylation (1). Each cell contains several hundred to thousand mitochondria and each of the mitochondria carry about 2 to 10 copies of mitochondrial DNA (mtDNA), resulting in approximately several hundred to thousand copies of mtDNA per cell. mtDNA is an approximately 16,000-bp circular double-stranded DNA molecule that is a prime target of oxidative damage due to its proximity to the electron transport chain (2). A higher rate of mutations occurs in the mtDNA than in nuclear DNA. This has been ascribed to the high rate of reactive oxygen species generated nearby, the lack of protective histones, and limited DNA repair capacity (3).

Several somatic mutations in the mtDNA have been observed in gastric tumors, including a very large deletion of 4,977 bp and mutations in the D-loop region (4, 5). The D-loop is a noncoding region of mtDNA that controls replication and transcription of mtDNA. Mutations in the D-loop may thus cause a decrease in mtDNA copy number or altered mtDNA gene expression (6). It has been hypothesized that mutations or decreases in mtDNA copy number could lead to a deficiency in oxidative phosphorylation and enhanced generation of ATP by glycolysis (6). *Heliobacter pylori* infection, one of the main risk factors for gastric cancer, also seems to induce mtDNA mutations in gastric cells (7, 8). Several studies have reported depletion in mtDNA copy number in gastric tumors compared with nontumorous gastric tissues (9–11).

Given the findings from experimental studies, we conducted a nested case–control study within a large

Authors' Affiliations: [1]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland; [2]Exposure Epidemiology and Risk Program, Harvard School of Public Health, Boston, Massachusetts; [3]Division of Epidemiology, Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt University, School of Medicine, Nashville, Tennessee; [4]Department of Epidemiology, Shanghai Cancer Institute, Shanghai, China; and [5]Department of Occupational and Environmental Health, University of Milan, Milan, Italy

Corresponding Author: Linda M. Liao, Occupational and Environmental Epidemiology Branch, National Cancer Institute, 6120 Executive Blvd, EPS 8003, MSC 7240, Bethesda, MD 20892. Phone: 301-451-5034; Fax: 301-402-1819; E-mail: donglm@mail.nih.gov

doi: 10.1158/1055-9965.EPI-11-0379

Published OnlineFirst July 22, 2011; DOI: 10.1158/1055-9965.EPI-11-0379

prospective cohort of women residing in Shanghai, China, to evaluate whether mtDNA copy number measured in leukocyte DNA could be a potential indicator of risk of developing gastric cancer.

Materials and Methods

Study population

The Shanghai Women's Health Study is a population-based prospective cohort study of women residing in Shanghai, China. A detailed description of the study methodology is available (12). In brief, 74,942 women aged 40 to 70 years residing in Shanghai were recruited into the study from 1996 to 2000. At baseline, detailed in-person interviews were conducted by trained interviewers to collect questionnaire information, yielding a response rate of 93%. Data were collected on demographic characteristics, personal habits, dietary habits, water drinking, physical activity, residential history, occupational history, family history of cancer, disease and surgery history, menstrual history, reproductive history and hormone use, and weight history. Body measurements were also taken at baseline. *H. pylori* infection was determined using *H. pylori* ELISA kits (Biohit ELISA kit) to detect serum IgG antibodies. In follow-up surveys, interviewers were able to interview and follow-up with 99.8% (2000–2002), 98.7% (2002–2004), and 96.7% (2004–2007) of cohort members or their next of kin.

Of the eligible participants, approximately 75% donated a blood sample at baseline. A 10 mL of blood sample was drawn into an EDTA Vacutainer tube. Samples were kept cold (0°C–4°C) and processed within 6 hours for long-term storage at −70°C. DNA was extracted from peripheral leukocytes by the standard phenol chloroform method. Included in this nested case–control study are 162 incident gastric cancer cases and 299 matched controls who provided a blood sample at the baseline survey. Incident gastric cancer cases were identified through in-person follow-up interviews and by linking to the Shanghai Cancer Registry and the Shanghai Vital Statistics Unit. Controls were randomly selected from cohort members and matched to cases by age at sample collection (±2 years), menopausal status, time of sample collection (morning or afternoon), date of sample collection (±1 month), and time interval since last meal (±2 hours). Controls were also free of any cancer at the time of cancer diagnosis for their corresponding case. Given this sample size, our study had sufficient power (80%) to detect a minimum OR of 2.1. Power calculations are based on a two-sided test with a type 1 error rate of $\alpha = 0.05$, comparing those in the highest quartile vs. those in the lowest quartile.

mtDNA copy number assay

Relative mtDNA copy number levels were measured in samples by a quantitative real-time PCR assay (13–15). In brief, relative mtDNA copy number is measured by determining the ratio of mitochondrial (mt) copy number to single-copy nuclear gene (S) copy number in experimental samples relative to reference DNA. The ratio is proportional to the mtDNA copy number in each cell. The single-copy nuclear gene used in this study was human hemoglobin β (*HBB*). Pooled DNA from 30 randomly selected Shanghai Women's Health Study participants served as the reference DNA pool used to create in every mt and S PCR run a fresh standard curve on which the system is calibrated, which ranged from 0.25 to 20 ng/μL. All PCRs were done on a 7900HT Fast Real-Time PCR system (Applied Biosystems). All samples were run in triplicate, and the average of all 3 measurements was calculated. Reasonably high reproducibility was observed with this assay with a coefficient of variance of 8% and an ICC of 81% on blinded duplicate samples.

Statistical analyses

Differences between cases and controls and levels of demographic and exposure characteristics were estimated using Wilcoxon nonparametric tests for continuous variables and ANOVA tests for categorical variables. To identify potential determinants of mtDNA copy number levels and/or factors that could modify the association between mtDNA copy number levels and gastric cancer risk, Wilcoxon nonparametric and ANOVA tests were used to evaluate differences among controls in relation to selected characteristics. The distribution of mtDNA copy number among controls was used to determine cutoff points for quartiles and median. Quartiles were collapsed into 2 categories, above and below the median, for stratified analyses because of small numbers. Conditional logistic regression, adjusted for potential risk factors for gastric cancer—age, body mass index (BMI: kg/m^2), education, fruit and vegetable intake, smoking status, recent nonsteroidal anti-inflammatory drug (NSAID) use, and family history of gastric cancer—was used to estimate ORs and 95% CIs. Tests for trend were calculated by modeling a variable coded 0, 1, 2, and 3. Sensitivity analyses included additional adjustments for *H. pylori* and stratified analysis by time between blood sample collection and cancer development. All analyses were conducted using SAS version 9.1 (SAS Institute).

Results

The characteristics of the 162 gastric cancer cases and 299 control subjects in this study are provided in Table 1. There were no significant differences between cases and controls for any of the selected characteristics. Few of the women smoked, drank alcohol, and used NSAID or multivitamins in this study. A large proportion of the women, both cases and controls, tested positive for *H. pylori* IgG antibodies. Overall, mtDNA copy number was not statistically different between gastric cancer cases and controls with medians of 1.04 and 1.06, respectively (Wilcoxon $P = 0.51$).

Published OnlineFirst July 22, 2011; DOI: 10.1158/1055-9965.EPI-11-0379

Table 1. Distribution of selected characteristics for women from the Shanghai Women's Health Study

Characteristics	Cases ($n = 162$)	Controls ($n = 299$)	*P*
Ever smoking, *n* (%)	9 (5.6)	13 (4.4)	0.56
Ever alcohol use, *n* (%)	3 (1.9)	6 (2.0)	0.91
BMI, kg/m^2, *n* (%)			
<25	88 (54.3)	186 (62.2)	
≥25 and <30	64 (39.5)	97 (32.4)	
≥30	10 (6.2)	16 (5.4)	0.26
Regular moderate or vigorous physical activity >3 MET h/d, *n* (%)	20 (26.3)	31 (24.2)	0.74
Education, *n* (%)			
High school or more	39 (24.1)	94 (31.4)	
Less than High school	123 (75.9)	205 (68.6)	0.10
Family history of gastric cancer, *n* (%)	14 (8.6)	19 (6.4)	0.36
NSAID use, within past year, *n* (%)	4 (2.5)	7 (2.3)	0.93
Regular multiple vitamin use, *n* (%)	11 (6.8)	22 (7.4)	0.82
H. pylori positive,[a] *n* (%)	129 (96.3)	226 (92.6)	0.16
Age, y, median (IQR)	61 (50–65)	61 (50–65)	0.80
Fruits/vegetables intake, g/d, median (IQR)	452.5 (297.2–635.1)	485.4 (348.6–697.7)	0.12
Fruits, g/d, median (IQR)	211.5 (92.4–320.3)	227.6 (120.4–352.4)	0.17
Vegetables, g/d, median (IQR)	242.1 (156.0–372.4)	250.0 (177.1–357.8)	0.32
Meat intake, g/d, median (IQR)	38.7 (21.7–59.4)	37.3 (23.9–56.3)	0.93
mtDNA copy number, median (IQR)	1.04 (0.87–1.25)	1.06 (0.88–1.29)	0.51

NOTE: Continuous variables are displayed as medians (IQR) and frequencies are displayed as counts (percentage). Comparison of cases and controls conducted using the χ^2 test for categorical variables and the Wilcoxon nonparametric test for continuous variables.

Abbreviation: MET, metabolic equivalent.

[a]Available on only 378 subjects.

We also evaluated whether mtDNA copy number levels differed by selected characteristics (Table 2). Overall, we found no differences in mtDNA copy number levels by smoking status, alcohol use, obesity, physical activity, family history of gastric cancer, NSAID or multivitamin use, *H. pylori* status, age, and fruit and vegetable or meat intake among the controls. However, several of these comparisons could be limited by small numbers in certain strata. When characteristics were evaluated continuously, only age was inversely correlated with mtDNA with a Spearman correlation of −0.13 ($P = 0.02$).

Overall, mtDNA copy number was not significantly associated with gastric cancer risk (Table 3). Compared with the highest quartile of mtDNA copy number, those with the lowest quartile of mtDNA copy number were associated with an OR of 1.05 (95% CI = 0.58–1.90; $P_{trend} = 0.69$). Additional adjustment for *H. pylori* status (one of the main risk factors for gastric cancer), which was only available on a subset of the samples ($n = 378$ subjects), did not change the effect estimates appreciably.

However, analyses stratified by the interval between blood collection and cancer diagnosis (reference date for controls) revealed a different pattern (Table 4). Among those diagnosed within 2 years of sample collection, low mtDNA was associated with an OR of 5.32 (95% CI = 1.03–27.60). Subsequent time periods did not reveal any significant associations between mtDNA copy number and risk of developing gastric cancer. After cases diagnosed within the first 2 years of blood sample collection were excluded, an association between mtDNA copy number and gastric cancer risk was no longer present.

Discussion

In this prospective study, we found no overall association between mtDNA copy number and risk of developing gastric cancer. However, a positive association between low mtDNA copy number in blood drawn within the 2 years prior to cancer diagnosis and risk of developing gastric cancer was observed. These findings suggest that low levels of mtDNA copy number could be an indicator of impending gastric cancer diagnosis, but our results are preliminary given our relatively small sample size.

mtDNA copy number variations in leukocyte DNA have been observed in several types of cancers (14, 16–19). Thus far, results from these 5 studies are somewhat mixed. The majority of these studies have reported an increased risk of developing cancer with higher mtDNA copy number but at varying definitions/levels of "high" mtDNA copy number. Xing et al. was the only study that

Published OnlineFirst July 22, 2011; DOI: 10.1158/1055-9965.EPI-11-0379

Table 2. mtDNA copy number levels by selected characteristics

Characteristic	Controls					
	n	Mean	25%	Median	75%	*P*
Smoking status						
Never	286	1.13	0.88	1.07	1.28	0.54
Ever	13	1.02	0.83	0.96	1.36	
Alcohol use						
Never	293	1.12	0.88	1.06	1.28	0.23
Ever	6	1.41	0.95	1.38	1.53	
BMI, kg/m^2						
<25	186	1.15	0.90	1.08	1.30	0.39
≥25 and <30	97	1.08	0.84	1.04	1.23	
≥ 30	16	1.09	0.90	1.01	1.36	
Regular moderate or vigorous physical activity						
≤3 MET h/d	97	1.12	0.86	1.05	1.27	0.58
>3 MET h/d	31	1.08	0.92	1.01	1.21	
Family history of gastric cancer						
No	280	1.13	0.88	1.06	1.29	0.45
Yes	19	1.13	0.97	1.08	1.39	
NSAID use, within past year						
No	292	1.12	0.88	1.06	1.29	0.27
Yes	7	1.23	1.05	1.16	1.30	
Regular multiple vitamin use						
No	277	1.13	0.88	1.06	1.28	0.78
Yes	22	1.11	0.91	1.16	1.31	
H. pylori positive[a]						
No	18	1.10	0.76	1.04	1.28	0.28
Yes	226	1.16	0.90	1.12	1.33	
Age at baseline, y						
<50	68	1.16	0.89	1.10	1.36	0.14
50–60	70	1.19	0.86	1.16	1.34	
>60	161	1.08	0.87	1.02	1.23	
Fruits/vegetables intake, g/d						
<Median	149	1.12	0.87	1.03	1.25	0.39
≥Median	150	1.13	0.89	1.08	1.31	

NOTE: *P* value comparing mtDNA copy number between different levels of characteristic (ANOVA and Wilcoxon).
Abbreviation: MET, metabolic equivalent.
[a]*H. pylori* status available on 244 controls only.

Table 3. ORs and 95% CIs for mtDNA copy number and gastric cancer

Quartile	Range	Cases	Controls	Unadjusted		Adjusted[a]		Adjusted[b]	
				OR	95% CI	OR	95% CI	OR	95% CI
4	≥1.29	33	62	1.00		1.00		1.00	
3	1.06–<1.29	38	62	0.91	0.52–1.59	0.91	0.51–1.60	1.02	0.54–1.91
2	0.88–<1.06	32	62	1.22	0.69–2.16	1.19	0.66–2.14	1.51	0.78–2.91
1	<0.88	31	63	1.09	0.61–1.95	1.05	0.58–1.90	1.25	0.65–2.40
P_{trend}					0.59		0.69		0.35

[a]Adjusted for smoking, age, BMI, fruit and vegetable intake, education, family history of gastric cancer, and NSAID use.
[b]Same adjustments as above but additionally adjusted for *H. pylori* status within 378 subjects (134 cases, 244 controls).

Published OnlineFirst July 22, 2011; DOI: 10.1158/1055-9965.EPI-11-0379

Table 4. Association between mtDNA copy number levels and risk of developing gastric cancer stratified by time between sample collection and cancer diagnosis

Time, y	Median	Range	Cases	Controls	OR[a]	95% CI
<2	2	≥1.06	10	32	1.00	
	1	<1.06	18	18	**5.32**	**1.03–27.60**
2–4	2	≥1.06	22	46	1.00	
	1	<1.06	26	41	1.61	0.64–4.06
4–6	2	≥1.06	19	32	1.00	
	1	<1.06	15	31	0.71	0.27–1.83
>6	2	≥1.06	23	39	1.00	
	1	<1.06	29	60	0.86	0.39–1.90

[a]Adjusted for smoking, age, BMI, fruit and vegetable intake, education, family history of gastric cancer, and NSAID use.

observed an association between lower mtDNA content and renal cell cancer risk (14). Three of these studies have been conducted within case–control studies and 2 studies were nested within prospective cohort studies (18, 19). The mechanism through which altered mtDNA copy number plays a role in carcinogenesis still remains unclear, but it has been shown in several studies that mtDNA depletion can alter mitochondrial gene expression and lead to a deficiency in oxidative phosphorylation and enhanced generation of ATP by glycolysis (11). The positive association we observed with lower levels of mtDNA copy number among cases diagnosed within 2 years of blood collection is consistent with the mtDNA depletion that has been reported in studies of gastric and other tumors (9–11, 20). However, to our knowledge, data are not available on the correlation between mtDNA copy number in leukocytes and gastric tissue.

The association we observed between low mtDNA copy number and increased risk of developing gastric cancer seems to fade as the time between blood sample collection and cancer diagnosis increases. As the association seems to be particularly strong among cases diagnosed within 2 years of blood sample collection, it is possible that the association may be driven by low mtDNA copy number among undiagnosed gastric cancers at the time of blood sample collection. One could postulate that in a retrospective case–control study setting, a similar association with what we observed among those diagnosed within 2 years of blood sample collection may be detected. The timing of when the blood sample was taken in relation to gastric cancer development seems to be an important issue in the measurement of mtDNA copy number.

To the best of our knowledge, this is the first study to evaluate mtDNA copy number measured in leukocyte DNA and risk of gastric cancer, as well as the first to examine the association prospectively by years between blood collection and cancer diagnosis. Strengths of our study include measurement of mtDNA copy number in triplicate and the collection of prospective blood samples from study subjects when they were healthy. In addition, there was a high proportion of follow-up (99.8%) and a large proportion of the cohort providing prediagnostic blood samples. Because of the relatively small size of our study, we were unable to evaluate potential interactions with other gastric cancer risk factors.

In conclusion, we observed no overall association between leukocyte mtDNA copy number levels and risk of developing gastric cancer. Through stratified analyses, we observed a possible indication of early disease with lower mtDNA copy number levels. As our study was limited by a small sample size, the relationship between mtDNA copy number and gastric cancer should be further explored in a larger study with prospective samples to confirm these findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors express their appreciation to the Shanghai residents who participated in the study and thank the research staff of the Shanghai Women's Health Study for their dedication and contributions to the study.

Grant Support

This research was supported by NIH research grant R37 CA70867, the Intramural Research Program contract N02 CP1101066, and the NIEHS grant P30ES000002.

Received April 20, 2011; revised June 10, 2011; accepted June 21, 2011; published OnlineFirst July 22, 2011.

References

1. Hatefi Y. The mitochondrial electron transport and oxidative phosphorylation system. Annu Rev Biochem 1985;54:1015–69.
2. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005;6:389–402.

Published OnlineFirst July 22, 2011; DOI: 10.1158/1055-9965.EPI-11-0379

3. Lee HC, Wei YH. Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging. Exp Biol Med 2007;232:592–606.
4. Hung WY, Wu CW, Yin PH, Chang CJ, Li AF, Chi CW, et al. Somatic mutations in mitochondrial genome and their potential roles in the progression of human gastric cancer. Biochim Biophys Acta 2010;1800:264–70.
5. Tuppen HA, Blakely EL, Turnbull DM, Taylor RW. Mitochondrial DNA mutations and human disease. Biochim Biophys Acta 2010;1797: 113–28.
6. Shadel GS. Expression and maintenance of mitochondrial DNA: new insights into human disease pathology. Am J Pathol 2008;172: 1445–56.
7. Machado AM, Figueiredo C, Touati E, Maximo V, Sousa S, Michel V, et al. *Helicobacter pylori* infection induces genetic instability of nuclear and mitochondrial DNA in gastric cells. Clin Cancer Res 2009;15: 2995–3002.
8. Machado AM, Figueiredo C, Seruca R, Rasmussen LJ. *Helicobacter pylori* infection generates genetic instability in gastric cells. Biochim Biophys Acta 2010;1806:58–65.
9. Li F, Wang X, Han C, Lin J. Decreased mtDNA copy number of gastric cancer: a new tumor marker?Chin J Clin Oncol 2004;1:250–5.
10. Wu CW, Yin PH, Hung WY, Li AF, Li SH, Chi CW, et al. Mitochondrial DNA mutations and mitochondrial DNA depletion in gastric cancer. Genes Chromosomes Cancer 2005;44:19–28.
11. Lee HC, Yin PH, Lin JC, Wu CC, Chen CY, Wu CW, et al. Mitochondrial genome instability and mtDNA depletion in human cancers. Ann N Y Acad Sci 2005;1042:109–22.
12. Zheng W, Chow WH, Yang G, Jin F, Rothman N, Blair A, et al. The Shanghai Women's Health Study: rationale, study design, and baseline characteristics. Am J Epidemiol 2005;162:1123–31.
13. Liu CS, Tsai CS, Kuo CL, Chen HW, Lii CK, Ma YS, et al. Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. Free Radic Res 2003;37:1307–17.
14. Xing J, Chen M, Wood CG, Lin J, Spitz MR, Ma J, et al. Mitochondrial DNA content: its genetic heritability and association with renal cell carcinoma. J Natl Cancer Inst 2008;100:1104–12.
15. Hou L, Zhu ZZ, Zhang X, Nordio F, Bonzini M, Schwartz J, et al. Airborne particulate matter and mitochondrial damage: a cross-sectional study. Environ Health 2010;9:48.
16. Shen J, Platek M, Mahasneh A, Ambrosone CB, Zhao H. Mitochondrial copy number and risk of breast cancer: a pilot study. Mitochondrion 2010;10:62–8.
17. Bonner MR, Shen M, Liu CS, Divita M, He X, Lan Q. Mitochondrial DNA content and lung cancer risk in Xuan Wei, China. Lung Cancer 2009;63:331–4.
18. Lan Q, Lim U, Liu CS, Weinstein SJ, Chanock S, Bonner MR, et al. A prospective study of mitochondrial DNA copy number and risk of non-Hodgkin lymphoma. Blood 2008;112:4247–9.
19. Hosgood HD III, Liu CS, Rothman N, Weinstein SJ, Bonner MR, Shen M, et al. Mitochondrial DNA copy number and lung cancer risk in a prospective cohort study. Carcinogenesis 2010;31:847–9.
20. Lee HC, Li SH, Lin JC, Wu CC, Yeh DC, Wei YH. Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma. Mutat Res 2004;547:71–8.

Cancer Epidemiology, Biomarkers & Prevention

AACR American Association for Cancer Research

Mitochondrial DNA Copy Number and Risk of Gastric Cancer: a Report from the Shanghai Women's Health Study

Linda M. Liao, Andrea Baccarelli, Xiao-Ou Shu, et al.

Cancer Epidemiol Biomarkers Prev 2011;20:1944-1949. Published OnlineFirst July 22, 2011.