

Short Communication

Detection of Cotinine in Newborn Dried Blood Spots

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Abstract

Maternal smoking while pregnant is a plausible risk factor for childhood cancers because many seem to initiate *in utero* and tobacco-specific carcinogens cross the placenta. Social desirability bias may affect maternal report of smoking in case-control studies and could explain inconsistently observed associations with offspring cancer. Detection of tobacco smoke biomarkers in dried blood spots (DBS), which are increasingly stored by newborn screening programs, may improve retrospective assessment of fetal tobacco exposure. As proof-of-principle, we examined cotinine in DBS of 20 infants enrolled in a pilot study of pregnancy among low-income women. We recruited 107 pregnant women (<30 weeks of gestation) from six Women, Infants, and Children clinics in Minneapolis and St. Paul in 1999. Blood samples obtained at enrollment were tested for total cotinine using gas chromatography/mass spec-

trometry. Women were then interviewed at 7 months of gestation to determine current smoking habits. DBS were obtained from the Minnesota Department of Health. We tested DBS from 10 infants whose mothers had detectable serum cotinine at baseline and 10 control infants whose mothers had none. One quarter of each DBS was assayed for cotinine using gas chromatography/mass spectrometry; levels were estimated assuming 50 μ L blood per sample. Mean cotinine was 29 ng/mL (SD, 7.5), 45 ng/mL (SD, 9.7), and 9 ng/mL (SD, 7.4), respectively, among infants of all smokers, infants of four women who acknowledged smoking at 7 months of gestation, and infants of nonsmokers. These results suggest that DBS analysis may identify infants of women who smoke throughout pregnancy. (Cancer Epidemiol Biomarkers Prev 2007; 16(9):1902–5)

Introduction

Accumulating evidence suggests that many childhood cancers are initiated *in utero*. This includes young age at diagnosis, occasional prenatal detection, and embryonal histology (1). Chromosomal translocations found in leukemia cells have also been “backtracked” to dried blood spots (DBS) collected at birth (2). Etiologic studies of childhood cancers thus frequently focus on maternal exposures during pregnancy.

Tobacco smoke is a plausible risk factor for childhood cancers, as tobacco-specific biomarkers (3) and carcinogens (4) are known to cross the human placenta. However, associations between maternal tobacco use while pregnant and offspring cancer during childhood have been observed for the most part inconsistently (5–7).

Although this may reflect true null associations, most investigations have used case-control designs. Recall or social desirability biases may therefore have influenced the results of studies that collected smoking data by maternal interview.

DBS are routinely collected shortly after birth to test for inborn errors of metabolism. Several state newborn screening programs also store DBS for >5 years and allow their release for research purposes (8). These specimens, which represent a biochemical snapshot taken at birth, may therefore be incorporated into studies of childhood cancer. Detection of tobacco smoke biomarkers in newborn DBS may improve assessment of fetal tobacco smoke exposure compared with maternal interview. As proof-of-principle, we examined the tobacco-specific biomarker cotinine (9) in DBS of 20 infants enrolled in a pilot study of pregnancy among low-income women.

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Materials and Methods

Data collection methods for this study, which were approved by the University of Minnesota Research Subjects’ Protection Programs Human Subjects Committee, have been described previously (10). Briefly, we recruited 107 pregnant women at <30 weeks of gestation

Table 1. Mean cotinine in DBS from infants of smoking and nonsmoking mothers

| Maternal smoking status | <i>n</i> | Mean DBS cotinine, ng/mL (SD) | <i>P</i> * |
|-------------------------|----------|-------------------------------|------------|
| Nonsmoker | 10 | 8.8 (7.4) | |
| Smoker | 10 | 28.7 (7.5) | 0.002 |
| Confirmed at 7 mo | 4 | 44.7 (9.7) | <0.001 |
| Not confirmed at 7 mo | 6 | 18.0 (7.8) | 0.034 |

**P* value from *t* test for two samples comparing DBS from infants of smokers with DBS from infants of nonsmokers.

from six Women, Infants, and Children clinics in Minneapolis and St. Paul between January and June of 1999. Participants provided blood samples at enrollment and were interviewed in person about demographics, smoking, and other health-related habits. They were unaware that their responses to questions about smoking would be validated. We interviewed participants by telephone about current smoking habits, among other items, at ~7 months of gestation and following birth. DBS of participants' newborn infants were obtained from the Minnesota Department of Health with maternal consent.

Blood samples taken at enrollment were tested for total cotinine using gas chromatography/mass spectrometry. Results of these analyses have been reported previously (10).

DBS were individually sealed and stored at 4°C until retrieval for analysis of cotinine in 2006. For this report, we tested DBS from 10 infants whose mothers had the highest detected levels of serum cotinine at baseline (range, 38–112 ng/mL) and from 10 infants randomly selected from among those whose mothers had no detectable serum cotinine (termed smoking and non-smoking mothers herein). We also tested a DBS from an adult smoker and blank filter paper as positive and negative controls, respectively. One quarter of each DBS was assayed by first solubilizing the spot by sonication in 0.5 mL of 0.2 N sodium hydroxide. The solution was then heated at 80°C for 1 h to hydrolyze cotinine from its *N*-glucuronide and [D_3]cotinine (20 ng) was added as internal standard. K_2CO_3 (0.5 mL of 25% aqueous) was added and the mixture was extracted with 1 mL methylene chloride. The methylene chloride layer was transferred to a 4-mL silanized vial and 25 μ L of methanol were added. It was gently blown down with nitrogen to ~100 μ , which was transferred to a 200- μ L glass insert vial. The solvent was gently blown down with nitrogen to ~25 μ and analyzed by gas chromatography/mass spectrometry as described (11).

Laboratory personnel were blinded to smoking status in all analyses.

Concentration of cotinine was calculated assuming 50 μ L blood per quarter DBS; test spotting in our laboratory indicated that 200 μ L fill the standard DBS collection circle. Cotinine levels are presented per estimated volume of whole blood, as the serum component was not isolated during DBS analysis. Mean cotinine concentration in DBS from infants with smoking and nonsmoking mothers was compared using the *t* test for two samples.

Results

Mean cotinine in DBS from infants of smoking and nonsmoking mothers is shown in Table 1 and Fig. 1. Cotinine concentration was significantly higher ($P = 0.002$) in DBS from infants of smokers (28.7 ng/mL; SD, 7.5) than in DBS from infants of nonsmokers (8.8 ng/mL; SD, 7.4). Four mothers reported smoking at 7 months of gestation; mean cotinine in DBS from their infants was 45 ng/mL (SD, 9.7). One mother reported not smoking at 7 months and five could not be reached; mean cotinine in DBS from their six infants was 18 ng/mL (SD, 7.8). Each of these means was significantly higher than that for infants of nonsmokers ($P < 0.001$ and $P = 0.034$, respectively).

Cotinine concentration in the DBS from an adult smoker was 435 ng/mL, whereas no cotinine was detected in blank filter paper.

Discussion

Case-control studies have comprised most etiologic research on maternal smoking as a risk factor for childhood cancer and likely will continue to do so. Childhood cancer is rare, with an annual rate of all cancers of about 150 per million children 0 to 14 years of age in the United States (12). Consequently, cohort studies are difficult to mount, and the few that have examined maternal tobacco use during pregnancy have been limited to studying either common diagnoses (13, 14) or all diagnoses combined (15, 16). Maternal tobacco use has occasionally been abstracted from birth records in retrospective studies (17). Although these data are collected without regard to later cancer, maternal smoking is substantially misclassified on birth certificates (18), which would attenuate relative risks. The remainder of case-control studies of childhood cancers have collected smoking data by maternal interview, raising the possibility of differential reporting between cases and controls, both due to the social disapproval of smoking while pregnant and feelings of culpability among mothers of cases (19). The results of our pilot study suggest that some of these issues may be circumvented by examining the tobacco-specific biomarker cotinine in

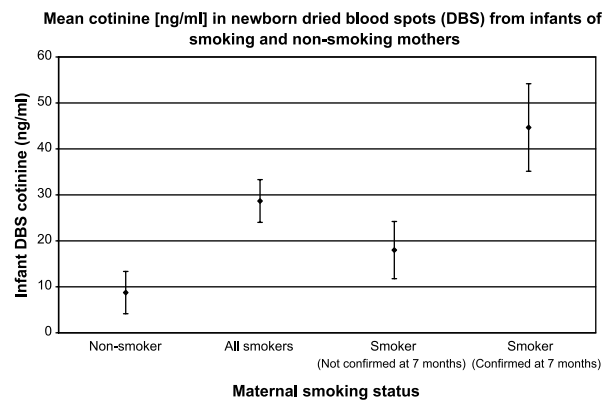


Figure 1. Mean cotinine (nanograms per milliliter) in newborn DBS from infants of smoking and nonsmoking mothers.

DBS obtained during the neonatal period and retrieved from storage.

We found that mean cotinine in newborn DBS of infants of smoking mothers was significantly higher than in those of infants of nonsmoking mothers. The highest levels were observed among infants of four mothers who confirmed continued smoking at 7 months of pregnancy. Cotinine was lower in DBS of infants of six mothers who did not confirm smoking at 7 months, some of whom may have ceased their habit after enrollment in the study, but still higher than in DBS of infants of nonsmokers. However, the presence of any cotinine in the DBS of the latter group requires explanation. Some mothers may have been misclassified as nonsmokers at enrollment if, for instance, they had temporarily ceased smoking. Other mothers may have lived or worked with smokers; unfortunately, we did not capture information about passive cigarette exposure. Last, we cannot rule out the possibility that DBS were contaminated during collection and transport to our facility.

The median half-life of cotinine was 28 h in infants in one study (20), and most DBS are collected 12 to 48 h after birth (21). Cotinine in DBS must therefore represent tobacco exposure shortly before delivery and, as might be expected, identifies babies born to the most refractory smokers. The Pregnancy Risk Assessment Monitoring System offers data on smoking cessation rates in a large, population-based study (22). In Pregnancy Risk Assessment Monitoring System, 57.5% of all pregnant women who smoked, but 78.1% of women who smoked >20 cigarettes daily, continued to smoke throughout pregnancy. The effect of information bias in maternal smoking status as determined by DBS cotinine analysis is unclear. Early pregnancy may be the relevant window of risk for some childhood cancers, in which case many subjects with fetal exposure to light cigarette smoking will be missed. However, any association of maternal smoking with offspring cancer is likely to be strongest among heavy smokers, whom our results suggest will be most often detected by DBS analysis. Tobacco-derived hemoglobin adducts have a longer half-life than does serum cotinine because erythrocytes persist for around 120 days and could conceivably identify mothers who smoked earlier in pregnancy (23). However, protein adduct assays require a relatively large amount of blood and may therefore be impractical for use with DBS (24).

Factors that may affect cotinine levels in DBS include storage, handling, and blood volume. Whereas DBS analyzed in this study were kept individually sealed at 4°C until analysis, storage conditions in many states differ (8); the extent to which cotinine in DBS may degrade under improper storage conditions is not known. It is also unclear whether cotinine may transfer between DBS stored adjacently without being individually sealed or whether handling of DBS by smokers could contaminate samples. Last, although we assumed a constant blood volume to calculate cotinine, in reality, the amount of blood may vary with spot size, punch location, and filter paper batch (25). We observed a 5-fold difference in DBS cotinine levels comparing newborns of confirmed smokers with those of confirmed nonsmokers; blood volume is unlikely to vary to a similar degree. In addition, the significant difference between cotinine levels in DBS from infants of smoking and nonsmoking mothers persisted in a sensitivity analysis assuming

60 and 40 µL blood per quarter DBS from each respective group (data not shown). Future analyses of cotinine in DBS may however quantify blood volume, for instance by using hemoglobin or albumen as standards.

In conclusion, newborn DBS may potentially be used to identify children whose mothers smoke throughout pregnancy but not those whose mothers ceased tobacco use earlier during gestation. This finding may be especially pertinent to case-control studies of childhood cancer, at least to determine fetal exposure to heavy maternal smoking. Despite this limitation, it is also possible that DBS verification of smoking may prompt more accurate report during maternal interview. The extent to which field conditions affect cotinine levels should be investigated and methods for obtaining DBS portions should be standardized, for instance by using punches. In addition, the cotinine assay should be optimized to expend a smaller portion of DBS than the quarter spot used in our experiment, as states may restrict the amount of stored DBS that can be given to researchers to preserve these precious nested biological samples.

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References

- Pizzo PA, Poplack DG, editors. Principles and practice of pediatric oncology. Philadelphia: Lippincott Williams and Wilkins; 2002.
- Greaves M. *In utero* origins of childhood leukaemia. *Early Hum Dev* 2005;81:123–9.
- Jauniaux E, Gulbis B, Acharya G, Thiry P, Rodeck C. Maternal tobacco exposure and cotinine levels in fetal fluids in the first half of pregnancy. *Obstet Gynecol* 1999;93:25–9.
- Milunsky A, Carmella SG, Ye M, Hecht SS. A tobacco-specific carcinogen in the fetus. *Prenat Diagn* 2000;20:307–10.
- Lightfoot TJ, Roman E. Causes of childhood leukaemia and lymphoma. *Toxicol Appl Pharmacol* 2004;199:104–17.
- Bunin GR. Nongenetic causes of childhood cancers: evidence from international variation, time trends, and risk factor studies. *Toxicol Appl Pharmacol* 2004;199:91–103.
- Little J. Epidemiology of childhood cancer. Lyon (France): IARC; 1999.
- Olney RS, Moore CA, Ojodu JA, Lindegren ML, Hannon WH. Storage and use of residual dried blood spots from state newborn screening programs. *J Pediatr* 2006;148:618–22.
- Bramer SL, Kallungal BA. Clinical considerations in study designs that use cotinine as a biomarker. *Biomarkers* 2003;8:187–203.
- Ross JA, Swensen AR, Murphy SE. Prevalence of cigarette smoking in pregnant women participating in the special supplemental nutrition programme for Women, Infants and Children (WIC) in Minneapolis and Saint Paul, Minnesota, USA. *Paediatr Perinat Epidemiol* 2002;16:246–8.
- Hecht SS, Carmella SG, Chen M, et al. Quantitation of urinary metabolites of a tobacco-specific lung carcinogen after smoking cessation. *Cancer Res* 1999;59:590–6.
- National Cancer Institute. SEER Cancer Statistics Review, 1975–2003. [accessed 2006 April 14]. Available from: http://seer.cancer.gov/csr/1975_2003/.
- Brooks DR, Mucci LA, Hatch EE, Cnattingius S. Maternal smoking during pregnancy and risk of brain tumors in the offspring. A prospective study of 1.4 million Swedish births. *Cancer Causes Control* 2004;15:997–1005.
- Mucci LA, Granath F, Cnattingius S. Maternal smoking and childhood leukemia and lymphoma risk among 1,440,542 Swedish children. *Cancer Epidemiol Biomarkers Prev* 2004;13:1528–33.
- Klebanoff MA, Clemens JD, Read JS. Maternal smoking during pregnancy and childhood cancer. *Am J Epidemiol* 1996;144:1028–33.
- Golding J, Paterson M, Kinlen LJ. Factors associated with childhood cancer in a national cohort study. *Br J Cancer* 1990;62:304–8.

17. Okcu MF, Goodman KJ, Carozza SE, et al. Birth weight, ethnicity, and occurrence of cancer in children: a population-based, incident case-control study in the State of Texas, USA. *Cancer Causes Control* 2002;13:595–602.
18. Northam S, Knapp TR. The reliability and validity of birth certificates. *J Obstet Gynecol Neonatal Nurs* 2006;35:3–12.
19. Higgins ST, Heil SH, Badger GJ, et al. Biochemical verification of smoking status in pregnant and recently postpartum women. *Exp Clin Psychopharmacol* 2007;15:58–66.
20. Leong JW, Dore ND, Shelley K, et al. The elimination half-life of urinary cotinine in children of tobacco-smoking mothers. *Pulm Pharmacol Ther* 1998;11:287–90.
21. National Newborn Screening Information System. Infant's age at time of initial testing in the U.S. in 2006. [accessed 2007 March 12]. Available from: <http://www2.uthscsa.edu/nnsis/>.
22. Colman GJ, Joyce T. Trends in smoking before, during, and after pregnancy in ten states. *Am J Prev Med* 2003;24:29–35.
23. Hecht SS. Tobacco carcinogens, their biomarkers, and tobacco-induced cancer. *Nat Rev Cancer* 2003;3:733–44.
24. Hecht SS, Carmella SG, Murphy SE. Tobacco-specific nitrosamine-hemoglobin adducts. *Methods Enzymol* 1994;231:657–67.
25. Mei JV, Alexander JR, Adam BW, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr* 2001;131:1631–6S.