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Tumor expression of environmental chemical-responsive genes and breast cancer mortality

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Abstract

Environmental phenols and phthalates are common ingredients in personal care products and some have been implicated in breast cancer progression. We have previously identified genes differentially expressed in response to low-dose exposure to diethyl phthalate (DEP) and methyl paraben (MPB) in a rat model. Herein we explore if these genes are associated with breast cancer mortality in humans. We profiled MPB- and DEP-responsive genes in tumors by NanoString® from a population-based cohort of 606 women with first primary breast cancer among whom 119 breast cancer-specific deaths occurred within 15+ years of follow-up. For each gene, Cox proportional hazards models were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). Results were validated in two publicly available datasets. The following results were obtained. From 107 DEP- and 77 MPB-responsive genes profiled, 44 and 30 genes, respectively, were significantly associated with breast cancer-specific mortality. Some top DEP-responsive genes are novel for breast cancer mortality, such as *ABHD14B* (for high-vs-low expression, HR 0.36, 95% CI: 0.2–0.5) and *TMC4* (HR 0.37, 95% CI: 0.3–0.5); top hits for MPB (*SLC40A1* (HR 0.37, 95% CI: 0.3–0.5) and *NTN4* (HR 0.39, 95% CI: 0.3–0.6)) are well-known predictors of breast cancer survival. *PLEKHA6* was another novel survival predictor, sensitive to hormonal receptor status (HR 0.5, 95% CI 0.3–0.9 for hormonal receptor-positive and HR 3.2, 95% CI 1.7–6.2 for -negative group). In conclusion, tumor expression of DEP- and MPB-responsive genes is associated with breast cancer mortality, supporting that exposure to these chemicals may influence the progression of breast cancer.

Key Words

- ▶ breast
- ▶ oncology
- ▶ mammary gland
- ▶ estrogen receptor

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Introduction

Low-molecular-weight phthalates and parabens are two classes of environmental chemicals widely used in personal care and medical products as plasticizers and solvents

(phthalates) or preservatives (parabens). Phthalate-based additives are known to leach from the plastic matrix and can end up in the environment and in humans

(Bosnir *et al.* 2003, Schettler 2006). Similarly, parabens, as antimicrobial agents, are directly added to food and pharmaceutical products (Soni *et al.* 2002). Many of these chemicals have shown to have endocrine-disruption properties which have the capacity of interfering the endocrine system and possibly playing a role in breast carcinogenesis (Delfosse *et al.* 2015). Humans are exposed to these chemicals through ingestion, inhalation and dermal contact. Neither phthalates nor parabens accumulate in the body and are rapidly enzymatically metabolized; phthalate diesters are hydrolyzed to the monoesters which are then absorbed and further oxidized (Albro & Lavenhar 1989, Silva *et al.* 2003), while parabens are metabolized to 4-hydroxybenzoic acid (PHBA) (Soni *et al.* 2001, 2002, Lakeram *et al.* 2006). Low-to-no acute toxicity or mutagenicity has been reported so far (Matthews *et al.* 1956), but some studies indicated estrogenic or anti-estrogenic activity for at least some phthalates (Jobling *et al.* 1995, Harris *et al.* 1997). These data suggest a potential role of parabens and phthalates in the development or progression of breast cancer (Darbre & Harvey 2014, Giulivo *et al.* 2016, Benjamin *et al.* 2017), but existing data on this subject are scarce, especially for humans.

Our study focuses on two specific phthalate and paraben congeners: diethyl phthalate (DEP, CAS # 84-66-2) and methyl paraben (MPB, CAS # 99-76-3) that are commonly used in personal care products. DEP is a low-molecular-weight phthalate widely used as a solvent in various consumer products, especially fragrance-containing ones (Api 2001). In the body, DEP is metabolized to mono-ethyl phthalate (MEP, CAS # 2306-33-4) which is readily detectable in urine. A study of Mexican women found positive associations between the highest concentrations of MEP and breast cancer risk (Lopez-Carrillo *et al.* 2010). However, we did not observe such association in our population-based Long Island Breast Cancer Caner Study Project (LIBCSP) (Parada *et al.* 2018). MPB is used, similarly to other parabens, as an antimicrobial agent in cosmetics, foods and pharmaceutical products. As the shortest member of the parabens, MPB displays the highest ability to penetrate the skin (Cosmetic Ingredient Review 2008); its urinary metabolite is PHBA, thus indistinguishable from other parabens. While earlier studies assumed extremely weak estrogenic activity of all parabens (Golden *et al.* 2005), there are several recent works demonstrating estrogenic effects of parabens (Darbre & Harvey 2014) including MPB, in both *in vitro* (Pugazhendhi *et al.* 2005) and *in vivo* (Sun *et al.* 2016) settings. Importantly, many

in vitro studies report carcinogenic effects of MPB (Wrobel & Gregoraszczyk 2013, Khanna *et al.* 2014, Lillo *et al.* 2017); at the same time, our recent population study did not find significant correlations between urinary levels of MPB and breast cancer incidence or mortality (Parada *et al.* 2019).

In our previous studies of a rat model, we found that low-dose exposure to MPB and DEP at critical windows of development induced changes in histology and development of mammary glands (Manservigi *et al.* 2015) and further identified a gene signature of exposure to low-dose DEP and MPB in the rat mammary glands: a set of genes was shown to be differentially expressed in rats exposed to low doses of MPB (Gopalakrishnan *et al.* 2017) and DEP (Gopalakrishnan K, Aushev VN, Teitelbaum SL, Manservigi F, Falcioni L, Panzacchi S, Belpoggi F, Parada H Jr, Garbowski G, Hibshoosh H, Santella RM, Gammon MD & Chen J, unpublished observations). In the current study, we aimed to examine whether the expression of these chemical-responsive genes in breast cancer tissue was associated with breast cancer mortality in a population-based study.

Materials and methods

Study population

We utilized resources from the follow-up component of the LIBCSP, which includes a population-based cohort of women newly diagnosed with first primary breast cancer who participated in the baseline interview within 2 to 3 months of diagnosis (Gammon *et al.* 2002) and were subsequently re-interviewed about 5 years later and followed for vital status (Cleveland *et al.* 2007). Participants were 1508 women newly diagnosed with first primary breast cancer between August 1, 1996 and July 31, 1997, who resided in Nassau and Suffolk counties of Long Island, NY. LIBCSP participants were predominantly (93%) Caucasian, and aged 25–98 (median age 58 years). Potentially eligible women were identified through daily or weekly contact with pathology/cytology departments of 33 collaborating institutions and confirmed by medical records review. The National Death Index (NDI) was used to ascertain all-cause and breast cancer-specific mortality among study participants, who were followed from diagnosis until December 31, 2014, for an average of 162 months (range 2.7–224 months, median 213 months) for the women included in this study. We obtained sufficient RNA for profiling from paraffin-embedded

tumor tissue from 616 of the 1508 patients; for the other LIBCSP patients, tumor slides were not made available by the diagnosing hospitals ($n=763$) or the extracted RNA was of insufficient/low quality ($n=139$); of these, 606 passed further QC. Demographics of both the overall patient cohort and the subset with sufficient tissue RNA are presented in a previously published study (Aushev *et al.* 2017). Among the 606 women with successfully profiled tumor tissue, 275 deaths occurred by the end of the follow-up period, of which 119 (43%) were related to breast cancer (ICD codes 174.9 and C-50.9 listed as a primary or secondary code on the death certificate). Consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used. The study protocol was approved by the Institutional Review Boards of the collaborating institutions.

Selection of MPB- and DEP-responsive genes

Discovery of MPB-responsive genes was previously published by Gopalakrishnan *et al.* (2017). Briefly, the MPB-responsive gene signature was identified based on differentially expressed genes in Sprague–Dawley rat mammary glands following low-dose MPB exposure during different windows of development. For pubertal MPB exposure, 295 genes were differentially expressed; after bootstrapping the samples and finding human orthologs, 77 genes (42 downregulated and 35 upregulated by MPB) were selected for further analysis in LIBCSP samples. Similarly, in DEP-exposed parous and nulliparous rats, 107 genes were selected as significantly differentially expressed (Gopalakrishnan K, Aushev VN, Teitelbaum SL, Manservigi F, Falcioni L, Panzacchi S, Belpoggi F, Parada H Jr, Garbowski G, Hibshoosh H, Santella RM, Gammon MD & Chen J, unpublished observations); importantly, all but one of these genes were downregulated by DEP exposure.

Tissue processing and RNA extraction

Formalin-fixed paraffin-embedded (FFPE) tumor sections from the population-based cohort were histopathologically reviewed by a trained pathologist and the cancer tissue was separated using manual microdissection (Rossner *et al.* 2009). Total RNA was extracted from tumor tissues using the Qiagen miRNeasy FFPE kit (Qiagen). RNA concentration and quality were determined with a NanoDrop spectrophotometer (Thermo Scientific). In total, 606 samples with sufficient RNA concentration were used in our analyses.

Gene expression profiling

Gene expression analysis of MPB- and DEP-responsive genes was performed with the NanoString nCounter platform (Seattle, WA, USA), using a custom-designed codeset and following the manufacturer's instructions. Briefly, 100 ng RNA was incubated with reporter and capture probes overnight at 65°C. Following hybridization, unbound probes were removed, and the purified complexes were aligned and immobilized on imaging cartridges using an nCounter Prep station. Code count detection was carried out by scanning cartridges in an nCounter Digital Analyzer to determine gene expression levels. As a quality control filter, we excluded all samples having less than 50,000 total counts for positive probes, or having less than $1.5 \times$ blank counts for housekeeping and endogenous genes. Raw counts were imported from RCC files with the NanoStringQCPro R package (version 1.6.0), background was subtracted as mean of negative control counts, geometric mean of housekeeping genes counts was used for normalization, and values were log-transformed for further analyses.

Coexpression of profiled genes was analyzed in R by building a covariance matrix; and principal component analysis was performed with 'pca' function of 'pcaMethods' package (Stacklies *et al.* 2007).

Molecular characteristics of tumors

Molecular characterization of the tumor tissues used in the current study is described in Aushev *et al.* (2017). Briefly, we determined hormone receptor status by measuring the expression of estrogen receptor (ER) and progesterone receptor (PR) genes, which was highly correlated with hormone receptor status determined by immunohistochemistry. Expression of the PAM50 panel of genes (Parker *et al.* 2009) was measured within the same NanoString panel and risk-of-recurrence score was determined as previously described in Aushev *et al.* (2017).

Survival analysis

Cox proportional hazards models (Cox 1972) were used for estimating the multivariable-adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for breast cancer-specific mortality associated with gene expression among the 606 women in our population-based cohort; non-breast cancer-specific deaths were censored. Survival analysis was performed using 'survival' package

in R (Borgan 2001). For each gene, range of cutoff thresholds (from second to eighth decile) was tested and the threshold with the lowest *P* value was used (Mihaly *et al.* 2013). For each gene, a Cox model was built using 'coxph' function with age and tumor stage (invasive or *in situ*) included as covariates. Multiple comparison adjustment was applied, where indicated, with the false discovery rate (FDR) threshold at 0.05.

All models were initially adjusted for age at diagnosis (continuous) and tumor stage. We further evaluated potential confounding using the methods described by Rothman and Greenland (Rothman *et al.* 2008) starting with a full multivariate model and using backward elimination. Potential confounders considered included: menopausal status (pre-/post-menopausal), family history of breast cancer in a first-degree relative, BMI at diagnosis, tumor size and education. If eliminating a covariate from the full Cox regression model changed the effect estimate by 10% or more, the covariate was considered a confounder and kept in the model. Otherwise that covariate was dropped from the multivariate model. None of the covariates tested met this criterion; thus, only results adjusted for age and tumor stage were presented. We also performed analyses stratified by ER/PR status (cases positive by the expression of at least one of the

receptors, hereafter named as ER/PR+ vs cases negative by both receptors, hereafter named as ER-/PR-). Kaplan-Meier survival (Kaplan & Meier 1958) plots were built for visualization of unadjusted associations between gene expression and breast cancer-specific mortality.

Results

From 77 MPB-responsive genes (42 downregulated and 35 upregulated by MPB) profiled in tumors, 30 genes (39%) were significantly (FDR ≤ 0.05) associated with breast cancer-specific survival (Fig. 1 and Supplementary Table 1, see section on [supplementary data](#) given at the end of this article). Increased expression of 17 of these genes was associated with lower breast cancer mortality, that is, longer survival. Strongest associations were found for *SLC40A1* (Ferroportin-1) (HR 0.4, 95% CI: 0.3–0.5) and *NTN4* (Netrin-4) (HR 0.4, 95% CI: 0.3–0.6), both are known breast cancer onco-suppressor genes. From those 17 genes associated with reduced breast cancer mortality, ten were downregulated by MPB in rats. For 13 remaining MPB-responsive genes, inverse associations were found, that is, their increased expression was associated with higher cancer mortality

Figure 1

Correlation of expression of 77 MPB-related genes with long-term breast cancer-specific survival of LIBCSP patients. (A) Volcano plot, showing age- and stage-adjusted Cox models, with each dot corresponding to a gene, horizontal axis displaying hazard ratio of high expression group comparing to low expression group (i.e. for the genes on the left higher expression is associated with better survival, genes on the right are associated with worse survival) and vertical axis displaying logarithm of *P* value of the given Cox model. (B) Survival curves for representative genes associated with longer (*SLC40A1*, *NTN4*) and shorter (*CHEK1*, *MCM2*) survival. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0357>.

(shorter survival), including established oncogenes *CHEK1* (HR 2.2, 95% CI: 1.5–3.2) and *MCM2* (HR 2.0, 95% CI: 1.4–2.9). Nine out of those 13 genes were upregulated by MPB in our rodent model.

Out of the 107 DEP-responsive genes tested (all but one of them were downregulated on DEP exposure), 44 genes (41%) were significantly ($FDR \leq 0.05$) associated with breast cancer mortality (Fig. 2 and Supplementary Table 2). From these 44 genes, 34 of them were associated with lower mortality, with prolactin receptor (*PRLR*), a known prognosis factor for low mortality, having the strongest association (for high-vs-low expression, HR 0.3, 95% CI: 0.2–0.5) with breast cancer-specific survival. Importantly, two other top hits have never been reported to be linked to breast cancer or progression: Abhydrolase Domain Containing 14B (*ABHD14B*) (HR 0.4, 95% CI: 0.2–0.5) and Transmembrane Channel Like 4 (*TMC4*) (HR 0.4, 95% CI: 0.3–0.5).

From the 606 tumor samples profiled, 80% were positive for ER or PR expression, as previously reported (Aushev *et al.* 2017). When the analyses were stratified by ER/PR status, it revealed that effects of some genes on breast cancer mortality were specific to the hormonal receptor status of the tumor. While the larger ($n=486$) hormone receptor-positive (ER/PR+) group revealed largely the same survival-associated genes found for the global population, in the hormone receptor negative (ER-/PR-) group ($n=120$), only two DEP genes (*MX1*, *PLEKHA6*) were strongly associated with breast cancer

survival ($FDR \leq 0.05$): reduced mortality for *MX1* (HR 0.3, 95% CI: 0.2–0.6) and increased mortality for *PLEKHA6* (HR 3.2, 95% CI: 1.7–6.2). Interestingly, associations of these genes with breast cancer survival demonstrated opposite trends in ER/PR+ group: strongly increased mortality on higher expression of *MX1* (HR 2.5, 95% CI: 1.6–3.9) and reduced mortality for *PLEKHA6* (HR 0.5, 95% CI: 0.3–0.9) (Fig. 3).

Besides ER/PR status, we were also able to measure expression of the genes from the PAM50 panel (Parker *et al.* 2009) and thus assess distribution of molecular subtypes in analyzed samples and corresponding survival (see Supplementary Fig. 5 of the previously published work; Aushev *et al.* 2017). The most common was Luminal A subtype ($n=179$), followed by Luminal B ($n=147$), Basal ($n=114$), Normal-like ($n=86$) and Her2-enriched ($n=80$). Because of low mortality in Luminal A and Normal-like groups (13 and 10 cases of breast cancer-associated deaths, respectively), we did not have enough statistical power to identify significant survival-associated genes in these groups separately, though the trends were similar. Thus, we combined Luminal A and Normal-like subgroups and confirmed the association for the top genes (*TMC4*: HR 0.2, 95% CI 0.1–0.5; *MX1*: HR 3.8, 95% CI 1.7–8.6; *IRF7*: HR 4.1, 95% CI 1.8–9.3). Although other subtypes, such as Basal, and Luminal B, displayed higher mortality, they constitute less common breast cancer subtypes. After adjustment for multiple comparison, only in the Her2-enriched subtype we identified two survival-related genes:

Figure 2

Correlation of expression of 107 DEP-related genes with breast cancer-specific survival; for legend see Fig. 1. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0357>.

Figure 3

Survival-associated genes specific for hormonal receptor (ER/PR) expression status. Four representative genes shown, data for all genes are in the Supplementary Table 3. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0357>.

EPCAM (HR 4.2, 95% CI: 2.0–8.9) and *QSOX1* (HR 4.2, 95% CI: 2.0–9.0). Complete results of survival analysis for all groups are available in Supplementary Table 3.

Using the PAM50 information and the algorithm (Parker *et al.* 2009) similar to the one used in clinical practice (Nielsen *et al.* 2014), we have estimated the risk-of-recurrence score for each patient of the LIBCSP cohort in our previous study (Aushev *et al.* 2017). This allowed us to re-analyze survival association of our genes of interest after stratification for the high- ($n=249$) and low/medium- ($n=357$) risk-of-recurrence groups. For the genes that co-expressed with the markers from PAM50 panels, we expected weaker association after this stratification. However, associations of top hits such as *TMC4* and *SLC40A1* remained significant in the low/medium-risk group: patients with low expression of these genes had high risk of recurrence despite being classified as low/medium risk by the conventional panel (Supplementary Fig. 2). For the PAM50-high-risk group, *PPAP2C* gene displayed the strongest association (HR 2.6, 95% CI: 1.7–4.1).

To validate our findings, we performed a similar survival analysis using two publicly available data sources – METABRIC and KMplot. The METABRIC dataset (Curtis *et al.* 2012) includes ~2000 breast cancer cases, with demographic and clinical characteristics similar to those of LIBCSP (predominantly Caucasian, 77% ER+). KMplot service (Gyorffy *et al.* 2010) combines multiple independent datasets including more than 1700 breast cancer cases.

As shown in Supplementary Fig. 1A, our most significant associations were reproducible in both METABRIC and KMplot datasets: genes correlated with better survival such as *SLC40A1*, *NTN*, *ABHD14B* – all remained significantly associated and with the same direction of association. ER/PR-status-specific associations were also largely confirmed: higher expression of *MX1* was associated with higher mortality in ER/PR-positive subgroup for all three datasets but lower mortality in ER/PR-negative subgroup (not significant in METABRIC dataset); the opposite association for *PLEKHA6* was confirmed in KMplot data (not significant in METABRIC) (Supplementary Fig. 1B). Supplementary Table 4 contains survival analysis data for the genes of interest available at METABRIC.

Discussion

In this study, we assessed the expression of MPB- and DEP-responsive genes, for their association with breast cancer-specific mortality. These genes were selected based on our previous works on animal model where we studied gene expression in mammary gland after the corresponding exposure. In the current work, we investigated associations between expression of these candidate genes in human breast tumors, with breast cancer mortality. We observed significant associations for ~40% of the genes examined. In both MPB and DEP gene sets, for the majority of the genes their higher expression was associated with increased

mortality. These associations were also largely confirmed in two independent datasets, METABRIC and KMplot.

The possible role of ED chemicals in breast cancer initiation and progression remains debatable. For MPB, early studies did not show a carcinogenic effect (Golden *et al.* 2005), and the estrogenic activity of parabens is considered extremely weak (Golden *et al.* 2005). For phthalates, estrogenic, anti-estrogenic or androgenic effects were reported in *in vitro* and animal studies but the results are often inconsistent and mostly obtained with high-dose exposure (Lovekamp-Swan & Davis 2003, Mckee *et al.* 2004). Our recent report did not show significant association between urinary phthalate levels and breast cancer incidence nor mortality (Parada *et al.* 2018). For parabens on the other hand, inverse associations with borderline significance was found (HR for Ln(methylparaben concentration): 0.92, 95% CI: 0.85–1.00, $P=0.05$; HR for Ln(propylparaben concentration): 0.93, 95% CI: 0.87–0.99, $P=0.03$) (Parada *et al.* 2019). In our previous animal study, we found that low-dose DEP and MPB exposure during certain windows of development induced significant morphological/histological and transcriptome changes in rat mammary glands (Manservigi *et al.* 2015, Gopalakrishnan *et al.* 2017). Our current study demonstrates that most of the genes that are, in a rat model, shown to be downregulated by DEP exposure (Gopalakrishnan *et al.* 2017) are associated with reduced breast cancer mortality (*PRLR*, *TMC4*, *ABHD14B*, *AR*, and others - see Fig. 2); these results are consistent with the 'oncogenic' effect of phthalate. For MPB, a similar trend is observed: MPB-downregulated *SLC40A1*, *NTN4*, *SLC1A1*, *PDGFRL*, and *ABCA9* are all mortality reducing, while MPB-upregulated *CHEK1* and *MCM2* are mortality enhancing (Fig. 1). However, a small number of genes showed the opposite trend including MPB-upregulated *SGK223*, *DHCR24*, *HSD17B7*, and *PPIL3* and DEP-downregulated *LCN2*, *EPCAM*, *ALDOC*, and *AADAT*. Therefore, we cannot make an unequivocal conclusion about oncogenic or onco-protective roles for these chemicals, reflecting the complex nature of environmental influence on breast carcinogenesis.

To our knowledge, this is the first study establishing a link between DEP- and MPB-responsive genes, and survival of breast cancer patients. We acknowledge certain limitations of this study. As in any translational research, results originated from animal experiments cannot be fully translated to humans; for the same reason, it is not surprising that not all the genes were concordant between the two characteristics we were interested in: association with survival on one hand and association with

MPB/DEP exposure on the other. Additional human studies are needed to assess this link more directly. At the same time, despite the limitation of animal-to-human translation, our findings on the association of these genes with breast cancer mortality, especially confirmed by two large independent datasets, are informative to the underlying mechanism. It is important to understand, however, that our observations are limited to certain types of tumors and/or populations. LIBCSP population consists of predominantly women of European ancestry, with 80% cases being hormonal receptor positive; all the strongest associations we found for this population overall did stand significant for ER/PR-positive subgroup, but not for the smaller subgroup of ER/PR-negative cases where other genes were revealed as strong predictors.

In terms of practical significance, it is important to mark the genes that allowed refining cases 'misclassified' by the conventional PAM50 panel: for example, *TMC4* alone was able to distinguish high-risk patients that were classified as low or medium risk.

Overall, we believe that our findings shed light on possible mechanism and identify previously unknown players in breast carcinogenesis and novel predictors of breast cancer mortality, which may potentially be valuable for clinical practice.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0357>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethics approval and consent to participate

For the Long Island Breast Cancer Study Project (LIBCSP), Institutional Review Board approval was obtained by all participating institutions and informed consent was obtained from all study participants; study was approved by the Institutional Review Board of Mount Sinai (Program for the Protection of Human Subjects).

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