Uterine Carcinomas in Tetrabromobisphenol A-Exposed Wistar Han Rats Harbor Increased *Tp53* Mutations and Mimic High-Grade Type I Endometrial Carcinomas in Women

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Abstract

Endometrial carcinoma is the most common gynecologic malignancy is the United States, and accounts for 6% of all cancers in women. The disease is classified as Type I or Type II based on clinicopathologic and molecular features. It is a multifactorial disease with a number of risk factors, including environmental exposures. How environmental exposures, such as flame retardants, may affect the incidence of endometrial cancer is a topic of current and ongoing interest. Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant found in a variety of household products. A recent two-year National Toxicology Program carcinogenicity study found that exposure to TBBPA was associated with a marked increase in the development of uterine tumors, specifically uterine carcinomas, in Wistar Han rats. Molecularly, TBBPA-induced uterine carcinomas in Wistar Han rats were characterized by a marked increase in *Tp53* mutation compared to spontaneous uterine carcinomas, as well as overexpression of *Her2*. Similar to spontaneous carcinomas, tumors in TBBPA-exposed rats were ERα positive and PR negative by immunohistochemistry. The morphologic and molecular features of uterine carcinomas in TBBPA-exposed rats resemble those of high-grade Type I tumors in women, and these data suggest that exposure to TBBPA may pose an increased cancer risk.

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Keywords

Tetrabromobisphenol A; endometrial carcinoma; *Tp53*; *Ctnnb1*; *Kras*; *Ccnd1*; *Her2*; *Cdh1*; Wistar Han rats

Introduction

Endometrial cancer is one of the leading causes of cancer morbidity and mortality in women in the United States, with an estimated 49,560 new cases and 8,190 deaths in 2013, and even higher estimates for 2014 (ACS 2013, 2014). Endometrial carcinoma (EC) in women is classified into two subtypes, Type I and Type II, based on clinicopathologic and molecular features. These two subtypes of EC have unique dualistic phenotypic and molecular features that correlate relatively well with the biologic behavior of these neoplasms (Horn *et al.*, 2007; Lax, 2004; Liu, 2007; Llobet *et al.*, 2009; Matias-Guiu *et al.*, 2001; Ryan *et al.*, 2005). Type I ECs, account for 80–90% of cases and, generally have a more favorable prognosis, while Type II ECs, which represent approximately 10–20% of cases, are associated with a poor prognosis. While the features of the two EC subtypes may overlap to some extent, subtyping is of particular utility when determining clinical outcome, prognosis, or therapeutic options for these tumors.

Based on clinicopathologic features, Type I tumors occur predominately in postmenopausal women, although they are also less frequently reported in premenopausal and perimenopausal women. Type I tumors are more often associated with nulliparity, are estrogen receptor-alpha (ERα) and progesterone receptor (PR) positive, and are slowly progressive with a lower risk of myometrial invasion (Mills and Longacre, 2011). Alternatively, Type II ECs occur in postmenopausal women, are associated with multiparity, and are high-grade with an aggressive clinical course. Morphologically, Type I tumors have endometrioid differentiation, characterized by irregularly shaped, variably sized glandular structures forming a merged, cribriform pattern or papillary proliferations, and associated with a desmoplastic stroma (Horn *et al.*, 2007). Type I ECs are typically low-grade and arise within a background of atypical hyperplasia, but high-grade Type I tumors do occur that behave more aggressively. Type II ECs (nonendometrioid) are much more poorly differentiated, characterized by tubulocystic, papillary, or solid forms composed of small, cuboidal to "hobnail" shaped cells with pleomorphic nuclei, frequent mitoses, and often bizarre nuclear forms. Necrosis may be common. Type II ECs are subdivided into a "clear cell" subtype based on cytoplasmic glycogen, or a "serous" subtype based on eosinophilic cytoplasm with apically located nuclei and focal cytoplasmic clearing. Features of both phenotypes may be present in the same tumor. Type II ECs arise within an estrogen-deficient, atrophic uterine epithelium and are associated with a precursor lesion of endometrial intraepithelial carcinoma. They are highly aggressive and infiltrative tumors, often spreading along the peritoneal cavity, and harbor a poor prognosis.

Aside from clinical and morphologic features, ECs in women can also be subclassified based on molecular phenotype (Horn *et al.*, 2007; Lax, 2004; Liu, 2007; Llobet *et al.*, 2009; Matias-Guiu *et al.*, 2001; Ryan *et al.*, 2005). Type I EC is associated with a higher incidence

of *PTEN* inactivation, microsatellite instability, and mutation of the *KRAS, CTNNB1*, and *PIK3CA* genes. Conversely, Type II EC is associated with a very low incidence of the above molecular alterations, but rather has a very high incidence of *TP53* mutation, as well as overexpression of Human Epidermal Growth Factor Receptor 2 (*HER2*), Cyclin D1 (*CCND1*) and Cyclins D1/E1 (*CCND/E1*), inactivation of *P16*, and alterations in E-cadherin (*CDH1*), expression.

While all Type II ECs are considered high-grade, an accepted and widely used histologic grading system has been developed for Type I ECs to distinguish more aggressive, high-grade Type I tumors from low-grade Type I tumors. High-grade Type I ECs have overlapping morphologic and molecular features of Type I and Type II tumors, including increased local invasion, loss of ERα and PR expression, increased KI67 protein expression, high rates of *TP53* mutation, and a poor prognosis, relative to low-grade Type I ECs (Liu, 2007; McConechy *et al*. 2012; Zannoni *et al*., 2010). Table 1 lists some of the clinicopathologic and molecular features of the subtypes of ECs (Horn *et al*., 2007; Liu, 2007; McConechy *et al*., 2012; 2001; Ryan *et al*., 2005; Zannoni *et al*., 2010).

A major risk factor for EC in women is hormone dysregulation. Many of the contributing factors to the development of EC influence estrogen levels, and increased estrogen is associated with EC development. Hormone (estrogen) therapy in menopausal women is associated with increased risk (Beral *et al*., 2005), whereas the use of oral contraceptives and multiparity are protective (Modan *et al*., 2001). Early menarche and late menopause are risk factors, and nulliparity is a risk factors, since pregnancy shifts the balance of hormones from estrogen to progesterone (Emons *et al*., 2000). Other risk factors include family history, diabetes, obesity, tamoxifen therapy, and ovarian pathologies such as granulosa cell tumors and polycystic ovarian syndrome, which are associated with unabated estrogen secretion (Emons *et al*., 2000). Aside from family history, the majority, if not all of these risk factors, are associated with hormone imbalance. It is becoming evident that an increasing number of compounds that people are exposed to in their environment, whether they are environmental pollutants or occupational compounds, have the ability to influence hormone production and function.

Tetrabromobisphenol A (TBBPA), a compound structurally related to Bisphenol A and formed by bromination of Bisphenol A, is a widely used flame retardant in electrical equipment, plastics, paper, furniture, and carpeting. It is a high volume production chemical in the United States, and the most widely used brominated flame retardant in the world (Schauer *et al*., 2006). It has been identified in fish, shellfish, and drinking water. Like Bisphenol A, TBBPA has been shown to have endocrine disrupting activity in vitro and in vivo, and in the uterotropic assay in ovariectomized mice, exposure to 500mg/kg TBBPA resulted in increased uterus:body weight ratios (Kitamura *et al*., 2005). It is known that estrogens, estrogenic chemicals, and estrogen agonists can act as tumor promoters as well as carcinogens (Vollmer, 2003). Previous studies have shown that TBBPA is possibly a very weak estrogenic agonist in vitro, but results have been equivocal (Hamers *et al*, 2006). It seems to stabilize and prevent turnover of estrogen levels through inhibition of estrogen sulfotransferase, a hormone metabolizing enzyme (Gosavi *et al*, 2013). However, there is little known about the long-term toxicologic and carcinogenic effects of TBBPA in humans.

In a recent National Toxicology Program (NTP) two-year carcinogenicity study, exposure of Wistar Han rats to TBBPA by corn-oil gavage was associated with a treatment-related increase in uterine carcinomas (NTP TR 587, 2013). Given the relevance of this compound to human exposures and the potential for endocrine disruption, investigation of the molecular mechanisms of uterine tumorigenesis due to TBBPA exposure in Wistar Han rats was warranted. The objective of this study was to characterize uterine carcinomas in Wistar Han rats exposed to TBBPA based on histopathologic and molecular criteria relevant to the human disease, to better understand the similarities and differences between human and rat EC, and to better understand the molecular events which occur in uterine carcinomas from TBBPA-treated Wistar Han rats.

Materials and Methods

Animals, histology, and immunohistochemistry

TBBPA-induced uterine carcinomas were obtained from Wistar Han [Crl:WI(Han)] rats in the two-year NTP bioassay. The care of animals on this study was according to National Institutes of Health (NIH) procedures as described in the " The U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals", available from the Office of Laboratory Animal Welfare, National Institutes of Health, Department of Health and Human Services, RKLI, Suite 360, MSC 7982, 6705 Rockledge Drive, Bethesda, MD 20892–7982 or online at http://grants.nih.gov/grants/olaw/olaw.htm#pol. The use of animals was approved by the NIEHS animal care and use committee. Forty-one formalin-fixed, paraffin-embedded (FFPE) uterine carcinomas from TBBPA-exposed animals were used in this study (10 low-dose, 15 mid-dose, 16 high-dose). A total of ten FFPE spontaneous carcinomas were available from control animals from various NTP studies. Due to the low incidence of spontaneously occurring uterine carcinomas in the current TBBPA carcinogenicity bioassay (two spontaneous tumors in corn-oil vehicle gavage control group), additional FFPE spontaneous tumors were obtained from other available Wistar Han rat carcinogenicity bioassay studies: one from polybrominated diethyl ether mixture (DE71) (corn-oil vehicle gavage), two from green tea extract (GTE-NTP TR 585, 2014) (deionized water vehicle), two from metal working fluids (MWF-NTP TR 586, 2014) (whole body inhalation), and three from antimony trioxide (AT) (whole body inhalation). Five FFPE normal uteri from corn-oil vehicle gavage control females in the concurrent TBBPA study were used as normal uterus controls. Four board-certified anatomic veterinary pathologists reviewed the histology of all uterine carcinomas using the standardized system of nomenclature and diagnostic criteria (SSNDC) guide for female reproductive proliferative lesions in rats to morphologically characterize the endometrial adenocarcinomas as well differentiated or poorly differentiated tumors (Table 2). If a tumor had areas that were well differentiated and poorly differentiated, then the tumor was classified as poorly differentiated. Tumors available for molecular biology included those > 0.5cm at the time of necropsy. These tumors were sectioned in half, and one half was flash frozen in liquid nitrogen for molecular analysis, and the other half fixed in 10% neutral buffered formalin (NBF) for histology and immunohistochemistry (IHC). Frozen tissue was available from six uterine carcinomas from TBBPA-exposed animals, and nine vehicle control animals from the above studies (one from TBBPA, two from GTE, three from AT, two from DE71, and one from MWF). The

above frozen tissues and paired FFPE tissues were used for *q*PCR and IHC analysis, respectively. For IHC, 5um paraffin-embedded sections were obtained and immunolabeled using the Vector ABC system with the following antibodies using routine methods: ERα (1:50, Beckman Coulter Inc., Fullerton CA), PR (1:50, Beckman Coulter Inc., Fullerton CA), TP53 (1:500, Vector Laboratories, Burlingame CA), and KI67 (1:100, Dakocytomation Corporation, Carpinteria CA). The expression of TP53 protein in uterine carcinomas was graded by a board-certified pathologist based on a severity grade of 0–3 (0 = no immunoreactivity, 1+ = minimal, 2+ = moderate, 3+ = strong immunoreactivity). KI67 was graded based on a scale of 0 = no immunoreactivity, 1+ = <25%, 2+ = 25–50%, and 3+ = >50% of tumor cell immunoreactivity.

DNA Isolation, PCR Amplification, and Autosequencing

DNA was isolated and extracted from 22 FFPE TBBPA-induced uterine carcinomas and ten FFPE spontaneous uterine carcinomas from rat control animals with DNeasy Tissue Kit (Qiagen, Valencia, CA). Amplification reactions were carried out by semi-nested polymerase chain reaction (PCR) using the designed primer sets (Table 3) for *Kras* exons 1 and 2 (codons 12, 13, 61), *Ctnnb1* exon 2 (corresponds to exon 3 in human), *Tp53* exons 5–8, and *Pten* exons 1–9 for 6 cDNA treated samples. Controls lacking DNA were run with all sets of reactions. PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR products were cycled with Terminal Ready Reaction Mix-Big Dye (Perkin Elmer, Foster City, CA), and the extension products were purified using the DyeEx 2.0 Spin Kit (Qiagen, Valencia, CA). The lyophilized PCR products were sequenced with an automatic sequencer (Perkin-Elmer ABI Model 3100). An electropherogram was used for comparison between normal uterus from vehicle controls and uterine carcinomas from vehicle controls and TBBPA-exposed animals.

qPCR—RNA was extracted and purified from frozen samples using the Invitrogen TRIzol Kit (Invitrogen, Carlsbad, CA). Fold increases and decreases in gene expression were determined by quantification of cDNA from target samples relative to a calibrator sample of controls. The 18s RNA gene was used as the endogenous control for normalization of initial RNA levels, and the expression of genes relevant for human EC (*P16, Her2, Ccnd1, Cdh1, Msh2, Msh3, Mlh1, Irf1, Pten*) was assessed.

Statistical Analysis

Incidences of neoplasms and non-neoplastic lesions were tested using one-sided poly-3 tests (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997). This method modifies the Cochran-Armitage linear trend test and Fisher's exact test to take length of survival into account, by more closely approximating the total number of animal-years at risk. The poly-3 trend test assesses the significance of the linear trend of incidences with dose, while the pairwise poly-3 tests assesses the significance of pairwise differences in incidences of each dose group compared to the control group. Differences are considered statistically significant if the one-sided p-value is less than 0.05.

Results

Exposure to TBBPA was associated with a treatment-related increase in uterine carcinomas in Wistar Han rats

The results of the two-year NTP chronic bioassay indicated that TBBPA exposure resulted in a statistically significant ($p < 0.05$) increase in the incidence of uterine carcinomas in the top 2 dose levels of female Wistar Han rats (Table 4). Based on histomorphologic criteria (Table 2), both TBBPA-induced (28 of 41, 68%) and spontaneous (7 of 10, 70%) uterine carcinomas in Wistar Han rats were classified as poorly differentiated, high-grade carcinomas, characterized by deeply infiltrative lobules, cords, and nests of poorly differentiated epithelial cells with marked cellular pleomorphism, atypia, high mitotic rate, and large foci of necrosis (Figure 1A–B and Table 5). The remaining carcinomas were considered fairly well-differentiated, as endometrial stromal invasion was limited to the superficial and mid-endometrium rather than replacing large areas of the deep stroma. Additionally, neoplastic glands exhibited milder nuclear atypia and pleomorphism as well as less necrosis (Figure 1C–D).

Uterine carcinomas in TBBPA-exposed Wistar Han rats show a tendency towards loss of PR expression and increased TP53 protein expression compared to spontaneous uterine carcinomas

Since criteria for classifying human Type I and II endometrial carcinoma relies partly on hormone (ERα/PR) expression, proliferation, and *Tp53* mutations, uterine carcinomas from TBBPA-exposed animals and spontaneous uterine carcinomas from vehicle controls and controls were examined for expression of ERα, PR, KI67, and *Tp53* by immunohistochemistry. All TBBPA-exposed (6 of 6, 100%) and spontaneous (9 of 9) uterine carcinomas in Wistar Han rats examined had diffuse positive nuclear immunoreactivity to antibodies against ERα. All spontaneous and TBBPA-exposed uterine carcinomas in animals had nuclear immunoreactivity in the endometrial stroma and myometrium, similar to the staining pattern seen in normal uteri. The neoplastic epithelial component in 2 of 6 uterine carcinomas in TBBPA-exposed animals had focal nuclear immunoreactivity for PR (Figure 2B), whereas in control animals, there was diffuse nuclear immunoreactivity in 6 of 9 tumors (Figure 2B inset). Poorly differentiated tumors from control and TBBPA exposed animals had increased nuclear immunoreactivity to KI67 (Figure 2C), whereas well-differentiated tumors had little to no KI67 expression (Figure 2D). Lastly, all examined uterine carcinomas (6 of 6) from TBBPA-exposed animals displayed diffuse and moderate to strong nuclear immunoreactivity to antibodies against TP53 (Figure 2E and Table 5). Five of nine spontaneous uterine carcinomas displayed nuclear immunoreactivity, with an average intensity score of 1.0; two displayed strong intensity (3+) and were associated with poor differentiation. Three spontaneous tumors had weak immunoreactivity (1+), and four were negative (Figure 2F). The two well-differentiated spontaneous uterine carcinomas did not express TP53.

Uterine carcinomas in TBBPA-exposed Wistar Han rats had increased incidence of *Tp53* mutations compared to spontaneous uterine carcinomas

Since mutations of several cancer genes are associated with clinical outcomes in humans, we screened spontaneous and TBBPA-associated uterine carcinomas for mutations relevant for EC in women. FFPE uterine carcinomas from TBBPA-exposed animals (n = 22) and spontaneous uterine carcinomas from vehicle controls (n = 10) were examined for mutations in *Kras, Ctnnb1*, and *Tp53* (Table 6). In TBBPA-exposed animals, 15 of 22 (68%) tumors harbored mutations in *Tp53*, often with multiple mutations per tumor (Table 7), whereas in spontaneous uterine carcinomas, 2 of 10 (20%) tumors had a mutation in this gene (Table 6). Of 22 uterine carcinomas from TBBPA-exposed animals that were assessed for mutations in *Ctnnb1* and *Kras*, 3 of 22 harbored mutations in *Ctnnb1*, and 1 of 22 animals had a *Kras* mutation (Table 6). Although, neither *Ctnnb1* nor *Kras* mutations were observed in spontaneous uterine carcinomas in this study (0 of 10) (Table 6) the incidence in uterine carcinomas from TBBPA-exposed rats was very small, and the relative differences in the number examined (10 spontaneous vs. 22 TBBPA-exposed) relatively large; therefore, this difference is likely not statistically significant, and may reflect variations in normal biology of these tumors in this strain.

Uterine carcinomas from TBBPA-exposed rats had increased *HER2* expression compared to spontaneous uterine carcinomas

Since alterations in gene expression of a variety of growth factors, oncogenes, and tumor suppressor genes are also present in ECs in women, including *HER2, CCND1*, and *CDH1*, we evaluated uterine carcinomas for alterations in the expression of these genes (Figure 3 and Table 7). All uterine carcinomas in TBBPA-exposed animals and vehicle control animals overexpressed *Her2*; expression in uterine carcinomas from TBBPA-exposed animals averaged a 30-fold increase compared to a 2-fold increase in spontaneous carcinomas in vehicle control rats. Approximately 40–80% of Type II tumors in humans overexpress *HER2*, as opposed to 10–30% of Type I EC tumors. All uterine carcinomas examined overexpressed *Ccnd1* and *Cdh1*, and the fold increase in expression was not significantly different between spontaneous uterine carcinomas and uterine carcinomas from TBBPA-exposed rats. In contrast to ECs in women, loss of *Pten* and *P16* tumor suppressor gene expression was not observed in spontaneous or TBBPA-associated carcinomas. In addition, both spontaneous and TBBPA-associated uterine carcinomas had an increased expression of microsatellite markers (*Msh2, Mlh1, Msh3*) instead of the characteristic decreased expression associated with microsatellite instability in ECs in women. Additionally, there was no difference in expression of *Irf1*, a tumor suppressor gene that has been shown to decrease with the grade of endometrial adenocarcinomas in women (Kuroboshi *et al.*, 2003) between TBBPA-induced and spontaneous tumors.

Discussion

Long-term exposure of Wistar Han rats to TBBPA resulted in treatment-related increased frequencies in uterine carcinomas. The majority of uterine carcinomas in both TBBPA-exposed animals and spontaneous tumors were considered poorly differentiated. Uterine carcinomas in this study varied from a relatively more well-differentiated glandular and

papillary form to poorly differentiated, infiltrative clusters, ribbons, or solid sheets of anaplastic glandular epithelial cells. A majority of poorly differentiated carcinomas also expressed high levels of KI67, in contrast to well-differentiated tumors. In women, KI67 expression is a prognostic factor correlated with higher grade, more aggressive ECs and a poor prognosis (Lax, 2004; Oreskovic *et al.*, 2004; Salvesen *et al.*, 1999; Zannoni *et al.*, 2010). In addition to similar morphologic features, for the most part alterations in gene expression and mutation spectra relevant for human EC were similar in Wistar Han rat uterine carcinomas regardless of TBBPA exposure. Uterine carcinomas in both groups had nuclear expression of ERα, increased expression of *Her2, Pten, P16, Irf1, Ccnd1* and *Cdh1*, and microsatellite markers. Loss of *Pten* and *P16* tumor suppressor gene expression are common events in Type I and Type II EC in women, respectively, and increased expression of *Ccnd1* is a common feature of Type II tumors. While EC in women is generally associated with microsatellite instability (loss of *Msh2, Mlh1, Msh3*) and loss of *Cdh1, P16*, and *Pten* function, both spontaneous and TBBPA-associated uterine carcinomas had increased expression of these mediators.

The primary molecular differences observed in uterine carcinomas from TBBPA-exposed animals compared to spontaneous tumors included a marked increase in *Tp53* mutation and increased *Her2* gene expression, and possibly a reduction in PR expression. While all uterine carcinomas, irrespective of differentiation or TBBPA exposure, showed nuclear expression of ERα, PR expression was reduced or lost in a majority of uterine carcinomas from TBBPA-exposed animals and fewer spontaneous tumors. Expression of ERα and loss of PR expression in TBBPA-exposed Wistar Han rats may suggest that these tumors remain estrogen responsive, but have lost receptor functionality for progesterone. Interestingly, research has shown that PR status may be a more reliable prognostic factor than ERα status in EC in women, and corresponds with a more aggressive, high-grade tumor (Oreskovic *et al.*, 2004; Zannoni *et al.*, 2010).

Spontaneous uterine carcinomas and carcinomas from TBBPA-exposed rats both shared morphologic and molecular features with Type I and II EC in women. Features common to both spontaneous and TBBPA-exposed tumors that were shared with Type I EC tumors in women included late onset, endometrioid differentiation, and ERα hormone receptor expression. Features shared between rat uterine carcinomas and human Type II EC include poor differentiation and deep invasion, high mitotic rate and KI67 index, increased cellular pleomorphism and atypia, *Her2* expression and *Tp53* mutation. While both spontaneous uterine carcinomas and those from TBBPA-exposed rats share features of Type I and Type II human EC, carcinomas from TBBPA-exposed rats have significant differences from spontaneous tumors. In addition to the above mentioned features, uterine carcinomas from TBBPA-exposed rats had significantly increased *Tp53* mutation and *Her2* expression compared to spontaneous tumors, features more often observed in Type II EC in women. Taken together, the morphologic and molecular features of uterine carcinomas in TBBPA-exposed rats overlap those of Type I and II EC in women, most consistent with high-grade Type I EC (Table 7), suggesting that uterine carcinomas in TBBPA-exposed rats may closely resemble this type of EC. However, many human EC tumors also share features of

both subtypes, and a clear distinction based on histomorphologic and molecular features is sometimes difficult.

By far, the most striking differences between uterine carcinomas in TBBPA-exposed animals and controls were the marked increase in *Her2* gene expression and *Tp53* mutation, two of the major genetic alterations in Type II EC in women (Liu, 2007). Human epidermal growth factor receptor 2 (*Her2* or *Her2/neu*) is a receptor tyrosine kinase that is an upstream regulator of a number of important cell proliferation and anti-apoptosis pathways. It is highly upregulated in Type II EC, and has been linked to adverse prognostic outcome and decreased overall survival (Doll *et al*., 2008). The *TP53* tumor suppressor gene is responsible for cell cycle checkpoint maintenance, apoptosis, and genomic stability (Blagosklonny, 2000). Loss of *TP53* tumor suppressor function is important in a wide variety of human cancers, including the development of aggressive endometrial cancers. *TP53* mutation is associated with high-grade tumors, advanced disease, and a poor prognosis (Liu, 2007). *TP53* mutations are extremely common in Type II EC (80–90%), and found in a subset (10–20%) of high-grade Type I EC in women (Liu, 2007; Llobet *et al*., 2009; Oreskovic *et al*., 2004; Zannoni *et al*., 2010); therefore, it is thought to possibly occur as a late event in the development of aggressive endometrial cancer. In this study, the high rate of *Tp53* mutation in uterine carcinomas from TBBPA-exposed rats compared to spontaneous tumors suggests that the increased incidence of uterine carcinomas in treated animals may be driven through a *Tp53*-dependent mechanism. While some degree of TP53 protein expression was observed in both spontaneous and TBBPA-associated uterine carcinomas, those tumors in TBBPA-exposed animals showed a much higher degree of immunoreactivity, consistent with the increase in mutation rate. The presence of nuclear immunoreactivity did not always correspond with the presence of a mutation in *Tp53* in this series of samples. However, these types of discordant results have been reported previously, and may be a result of gain of function of the *TP53* gene, accumulation of wildtype protein due to DNA damage (Blagosklonny, 2000), or a reflection of a low percentage of mutation-positive tumor cells (Bian *et al*., 2001; George *et al*., 2007; Kropveld *et al*., 1996). This latter explanation could also account for nuclear expression of TP53 in spontaneous samples, which was much lower than that of the TBBPA samples, in which there was a high rate of mutation. Alternatively, gene mutations may have occurred in unsequenced exons.

Other features of Type I and II EC in women include *Ctnnb1* and *Kras* mutation. Beta-catenin is the protein product of *Ctnnb1* and is an essential part of the WNT signaling pathway, regulating cellular processes including proliferation, motility, and survival (Wu *et al*., 2007). *CTNNB1* mutation in EC in women occurs in approximately 20–40% of Type I tumors compared to 0–5% in Type II tumors (Llobet *et al*., 2009). *KRAS* mutation, seen in 10–30% of Type I EC in women, (Llobet *et al*., 2009), is associated with constitutive activation, enhanced cellular proliferation, transformation, and cell survival, and has been associated with adverse clinical outcomes in women (Matias-Guiu *et al*., 2001). *Ctnnb1* and *Kras* mutations were not observed to play a significant role in either spontaneous uterine carcinomas or those from TBBPA-exposed animals in this study; the general lack of these mutations suggests that these are not critical pathways for the generation of these tumors in the Wistar Han rat model.

The results of this study suggest that uterine carcinomas in TBBPA-exposed Wistar Han rats most closely resemble high-grade Type I EC in women on the molecular level, which are typified in humans by conservation of ERα expression, a high rate of *TP53* mutation, more aggressive clinical course and a poor prognosis compared to other typical low-grade Type I tumors (Liu, 2007; McConechy *et al.*, 2012; Zannoni *et al.*, 2010). Interestingly, the BDII Han rat model develops a high rate of spontaneous uterine carcinomas (~90%) which, based on molecular features, have also been classified as similar to high-grade Type I ECs in women (Adamovic *et al.*, 2008; Behboudi *et al.*, 2001; Nordlander *et al.*, 2005; Samuelson *et al.*, 2009). They have an early onset, endometrioid differentiation, and are estrogen responsive, but have high rates of *Tp53* mutation, similar to the uterine carcinomas observed in TBBPA-exposed Wistar Han rats in this study. However, carcinomas in TBBPA-exposed rats have additional features of Type II ECs, including overexpression of *Her2*.

In summary, uterine carcinomas were observed with a higher frequency in Wistar Han rats exposed to TBBPA, have higher rates of proliferation, an increased rate of *Tp53* mutation, a tendency toward decreased PR expression, and increased *HER2* expression compared to spontaneous uterine carcinomas in Wistar Han rats. These features overlap with those of Type I and II EC in women, and are most concordant with high-grade Type I tumors, which are clinically more aggressive and harbor a poorer prognosis compared to other Type I tumors. Furthermore, the presence of such a high rate of *Tp53* mutations in TBBPA-exposed animals compared to controls suggests that uterine carcinogenesis in Wistar Han rats exposed to TBBPA is partially *Tp53*-dependent. Since alteration of *TP53* is relevant for aggressive endometrial cancer in women, and plays a predominant role in the pathogenesis of a number of other cancers in humans, it is reasonable to hypothesize that TBBPA exposures may pose a cancer risk to humans through similar mechanisms. However, inherent species differences in the metabolism of TBBPA or sensitivity to its biologic effects on uterine pathophysiology may exist, and further functional studies on the carcinogenic effects of TBBPA on the female reproductive tract are warranted.

Acknowledgements

Abbreviations

TBBPA	Tetrabromobisphenol A
EC	Endometrial carcinoma
ERα	Estrogen receptor-alpha

PR	Progesterone receptor
HER2	Human Epidermal Growth Factor Receptor 2
CCND/E1	Cyclins D1/E1
PTEN	Phosphatase and tensin homolog
P16	Cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1
CDH1	E-cadherin
Tp53	Tumor protein 53
Ctnnb1	Catenin (cadherin-associated protein), beta 1
Kras	Kirsten rat sarcoma viral oncogene homolog
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
NTP	National Toxicology Program
FFPE	Formalin-fixed, paraffin-embedded
DE71	Polybrominated diethyl ether mixture
AT	Antimony trioxide
SSNDC	Standardized system of nomenclature and diagnostic criteria
Msh2/Msh3	MutS protein homolog 2/3
Mlh1	MutL homolog 1
Irf1	Interferon regulatory factor 1

References

American Cancer Society: Cancer Facts and Figures. Atlanta, Ga: American Cancer Society; 2013.

American Cancer Society: Cancer Facts and Figures. Atlanta, Ga: American Cancer Society; 2014. http://www.cancer.org/cancer/endometrialcancer/detailedguide/endometrial-uterine-cancer-key-statistics

Adamovic T, Hamta A, Roshani L, Lu X, Rohme D, Helou K, Klinga-Levan K, Levan G. Rearrangement and allelic imbalance on chromosome 5 leads to homozygous deletions in the CDKN2A/2B tumor suppressor gene region in rat endometrial cancer. Cancer genetics and cytogenetics. 2008; 184:9–21. [PubMed: 18558284]

Bailer AJ, Portier CJ. Effects of treatment-induced mortality and tumor-induced mortality on tests for carcinogenicity in small samples. Biometrics. 1988; 44:417–431. [PubMed: 3390507]

Behboudi A, Levan G, Hedrich HJ, Klinga-Levan K. High-density marker loss of heterozygosity analysis of rat chromosome 10 in endometrial adenocarcinoma. Genes Chromosomes Cancer. 2001; 32:330–341. [PubMed: 11746974]

Beral V, Bull D, Reeves G. Endometrial cancer and hormone-replacement therapy in the Million Women Study. Lancet. 2005; 365:1543–1551. [PubMed: 15866308]

Bian Y-S, Osterheld M-C, Bosman FT, Benhattar J, Fontolliet C. p53 Gene Mutation and Protein Accumulation during Neoplastic Progression in Barrett's Esophagus. Mod Pathol. 2001; 14:397–403. [PubMed: 11353048]

Blagosklonny MV. p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect. Faseb J. 2000; 14:1901–1907. [PubMed: 11023974]

Dixon, D.; Leininger, JR.; Valerio, MG.; Johnson, AN.; Stabinski, LG.; Firth, CH. Proliferative lesions in the ovary, uterus, vagina, cervix, and oviduct in rats. Washington, DC: Guides for Toxicologic Pathology, STP/ARP/AFIP; 1999. p. 1-32.

Dixon D, Alison R, Bach U, Colman K, Foley GL, Harleman J, Haworth R, Herbert Heuser A, Long G, Mirsky M, Regan K, van Esch E, Westwood FR, Vidal J, Yoshida M. INHAND: Nonproliferative and Proliferative Lesions of the Rat and Mouse Female Reproductive Tract. J Toxicol. Pathol. 2014 In Press.

Doll A, Abal M, Rigau M, Monge M, Gonzalez M, Demajo S, Colas E, Llaurado M, Alazzouzi H, Planaguma J, Lohmann MA, Garcia J, Castellvi S, Ramon y Cajal J, Gil-Moreno A, Xercavins J, Alameda F, Reventos J. Novel molecular profiles of endometrial cancer-new light through old windows. J Steroid Biochem Mol Biol. 2008; 108:221–229. [PubMed: 18061438]

Emons G, Fleckenstein G, Hinney B, Huschmand A, Heyl W. Hormonal interactions in endometrial cancer. Endocr Relat Cancer. 2000; 7:227–242. [PubMed: 11174845]

George B, Datar RH, Wu L, Cai J, Patten N, Beil SJ, Groshen S, Stein J, Skinner D, Jones PA, Cote RJ. p53 gene and protein status: the role of p53 alterations in predicting outcome in patients with bladder cancer. J Clin Oncol. 2007; 25:5352–5358. [PubMed: 18048815]

Gosavi RA, Knudsen GA, Birnbaum LS, Pedersen LC. Mimicking of estradiol binding by flame retardants and their metabolites: a crystallographic analysis. Environ Health Perspect. 2013; 121:1194–1199. [PubMed: 23959441]

Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MH, Andersson PL, Legler J, Brouwer A. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol Sci. 2006; 92:157–173. [PubMed: 16601080]

Horn LC, Meinel A, Handzel R, Einenkel J. Histopathology of endometrial hyperplasia and endometrial carcinoma: an update. Ann Diagn Pathol. 2007; 11:297–311. [PubMed: 17630117]

Kitamura S, Suzuki T, Sanoh S, Kohta R, Jinno N, Sugihara K, Yoshihara S, Fujimoto N, Watanabe H, Ohta S. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci. 2005; 84:249–259. [PubMed: 15635150]

Kuroboshi H, Okubo T, Kitaya K, Nakayama T, Daikoku N, Fushiki S, Honjo H. Interferon regulatory factor-1 expression in human uterine endometrial carcinoma. Gynecologic Oncology. 2003; 91:354–358. [PubMed: 14599866]

Kropveld A, van Mansfeld ADM, Nabben N, Hordijk GJ, Slootweg PJ. Discordance of p53 status in matched primary tumours and metastases in head and neck squamous cell carcinoma patients. European Journal of Cancer. Part B: Oral Oncology. 1996; 32:388–393.

Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. Virchows Arch. 2004; 444:213–223. [PubMed: 14747944]

Liu FS. Molecular carcinogenesis of endometrial cancer. Taiwan J Obstet Gynecol. 2007; 46:26–32. [PubMed: 17389185]

Llobet D, Pallares J, Yeramian A, Santacana M, Eritja N, Velasco A, Dolcet X, Matias-Guiu X. Molecular pathology of endometrial carcinoma: practical aspects from the diagnostic and therapeutic viewpoints. Journal of clinical pathology. 2009; 62:777–785. [PubMed: 18977806]

Matias-Guiu X, Catasus L, Bussaglia E, Lagarda H, Garcia A, Pons C, Munoz J, Arguelles R, Machin P, Prat J. Molecular pathology of endometrial hyperplasia and carcinoma. Hum Pathol. 2001; 32:569–577. [PubMed: 11431710]

McConechy MK, Ding J, Cheang MC, Wiegand KC, Senz J, Tone AA, Yang W, Prentice LM, Tse K, Zeng T, McDonald H, Schmidt AP, Mutch DG, McAlpine JN, Hirst M, Shah SP, Lee CH, Goodfellow PJ, Gilks CB, Huntsman DG. Use of mutation profiles to refine the classification of endometrial carcinomas. J Pathol. 2012; 228:20–30. [PubMed: 22653804]

Mills AM, Longacre TA. Atypical endometrial hyperplasia and well differentiated endometrioid adenocarcinoma of the uterine corpus. Surg Pathol. 2011; 4:149–198.

Mittal KR, Barwick KW. Diffusely infiltrating adenocarcinoma of the endometrium. A subtype with poor prognosis. Am J Surg Pathol. 1988; 12:754–758. [PubMed: 3421412]

Modan B, Hartge P, Hirsh-Yechezkel G, Chetrit A, Lubin F, Beller U, Ben-Baruch G, Fishman A, Menczer J, Struewing JP, Tucker MA, Wacholder S. Parity, oral contraceptives, and the risk of

ovarian cancer among carriers and noncarriers of a BRCA1 or BRCA2 mutation. N Engl J Med. 2001; 345:235–240. [PubMed: 11474660]

Nordlander C, Behboudi A, Levan G, Levan KK. Allelic imbalance on chromosome 10 in rat endometrial adenocarcinomas. Cancer genetics and cytogenetics. 2005; 156:158–166. [PubMed: 15642397]

NTP. Toxicology studies of tetrabromobisphenol a (CAS No. 79–94-7) in f344/ntac rats and b6c3f1/n mice and toxicology and carcinogenesis studies of tetrabromobisphenol a in wistar han [crl:wi(han)] rats and b6c3f1/n mice (gavage studies). 2013. NTP Technical Report Series: http://ntp.niehs.nih.gov/ntp/about_ntp/trpanel/2013/october/draft_tr-587.pdf

NTP. Toxicology studies of green tea extract in f344/ntac rats and b6c3f1/n mice and toxicology and carcinogenesis studies of green tea extract in wistar han[crl:wi(han)] rats and b6c3f1/n mice (gavage studies). 2014. NTP Technical Report Series: http://ntp.niehs.nih.gov/?objectid=BD499CD4-123F-7908-7B1F22BDE9A5D1D7

NTP. Toxicology studies of antimony trioxide 10676-v (CASRN: 1309–64-4) in wistar han [crl:wi(han)] rats and b6c3f1/n mice and toxicology and carcinogenesis studies of antimony trioxide 10676-v in wistar han[crl:wi(han)] rats and b6c3f1/n mice (inhalation studies). 2014. NTP Technical Report Series: http://ntp.niehs.nih.gov/?objectid=BCD52E6A-123F-7908-7BD5298A5D912AF9

NTP. Toxicology studies of cimstar 3800 in f344/ntac rats and b6c3f1/n mice and toxicology and carcinogenesis studies of cimstar 3800 in wistar han [crl:wi (han)] rats and b6c3f1/n mice (inhalation studies). NTP Technical Report Series: http://ntp.niehs.nih.gov/ntp/about_ntp/trpanel/2014/may/draft_tr586_508.pdf#search=cimstar%2 03800. 2014.

NTP. Toxicology studies of pentabromodiphenyl oxide (technical) (DE 71) - M20287 (CASRN: 32534–81-9) in f344/n rats and b6c3f1/n mice and toxicology and carcinogenesis studies of pentabromodiphenyl oxide (technical) (DE 71) - M20287 in wistar han [crl:wi(han)] rats and b6c3f1/n mice (gavage studies). 2014. NTP Technical Report Series: http://ntp.niehs.nih.gov/?objectid=BD73BA18-123F-7908-7BD4AEF6AB4318BF

Oreskovic S, Babic D, Kalafatic D, Barisic D, Beketic-Oreskovic L. A significance of immunohistochemical determination of steroid receptors, cell proliferation factor Ki-67 and protein p53 in endometrial carcinoma. Gynecol Oncol. 2004; 93:34–40. [PubMed: 15047211]

Piegorsch, WW.; Bailer, AJ. Statistics for Environmental Biology and Toxicology, Section 6.3.2. London: Chapman and Hall; 1997.

Portier CJ, Bailer AJ. Testing for increased carcinogenicity using a survival-adjusted quantal response test. Fundamental and Applied Toxicology. 1989; 12:731–737. [PubMed: 2744275]

Ryan AJ, Susil B, Jobling TW, Oehler MK. Endometrial cancer. Cell and tissue research. 2005; 322:53–61. [PubMed: 15947972]

Salvesen HB, Iversen OE, Akslen LA. Prognostic significance of angiogenesis and Ki-67, p53, and p21 expression: a population-based endometrial carcinoma study. J Clin Oncol. 1999; 17:1382–1390. [PubMed: 10334522]

Samuelson E, Hedberg C, Nilsson S, Behboudi A. Molecular classification of spontaneous endometrial adenocarcinomas in BDII rats. Endocr Relat Cancer. 2009; 16:99–111. [PubMed: 19075038]

Schauer UM, Volkel W, Dekant W. Toxicokinetics of tetrabromobisphenol a in humans and rats after oral administration. Toxicol Sci. 2006; 91:49–58. [PubMed: 16481339]

Vollmer G. Endometrial cancer:experimental models useful for studies on molecular aspects of endometrial cancer and carcinogenesis. Endocr Relat Cancer. 2003; 10:23–42. [PubMed: 12653669]

Wu R, Hendrix-Lucas N, Kuick R, Zhai Y, Schwartz DR, Akyol A, Hanash S, Misek DE, Katabuchi H, Williams BO, Fearon ER, Cho KR. Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/beta-catenin and PI3K/Pten signaling pathways. Cancer cell. 2007; 11:321–333. [PubMed: 17418409]

Zannoni GF, Vellone VG, Arena V, Prisco MG, Scambia G, Carbone A, Gallo D. Does high-grade endometrioid carcinoma (grade 3 FIGO) belong to type I or type II endometrial cancer? A clinical-pathological and immunohistochemical study. Virchows Arch. 2010; 457:27–34. [PubMed: 20552366]
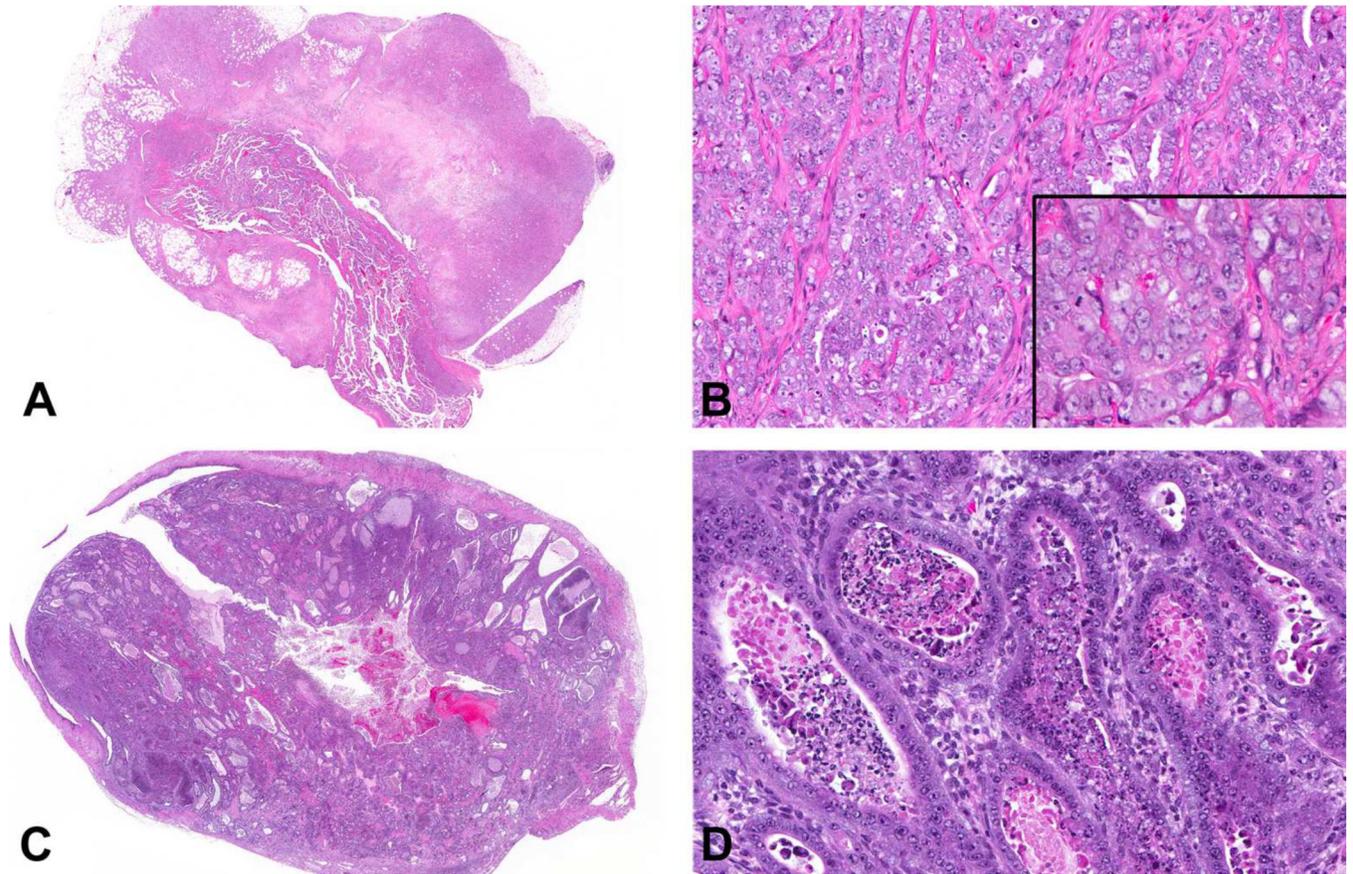
Figure 1.
Uterine carcinomas from TBBPA-exposed Wistar Han rats. (A) Poorly differentiated uterine carcinoma characterized by marked invasion through the uterine wall into the peri-uterine tissues, accompanied by large zones of necrosis. (B) Poorly differentiated uterine carcinomas were characterized by solid clusters, cords and nests of poorly differentiated epithelial cells with marked pleomorphism and cellular atypia, including large vesicular nuclei, multiple nucleoli, marked anisocytosis and anisokaryosis (inset), and mitotic figures. (C) Well-differentiated carcinomas were fairly well circumscribed with stromal invasion relatively limited to the superficial and mid-endometrium rather than replacing large areas of the deep stroma, and a glandular phenotype. (D) Glands were generally lined by one or more layers of fairly well-differentiated cuboidal to columnar epithelial cells with minimal to mild pleomorphism and few mitotic figures.
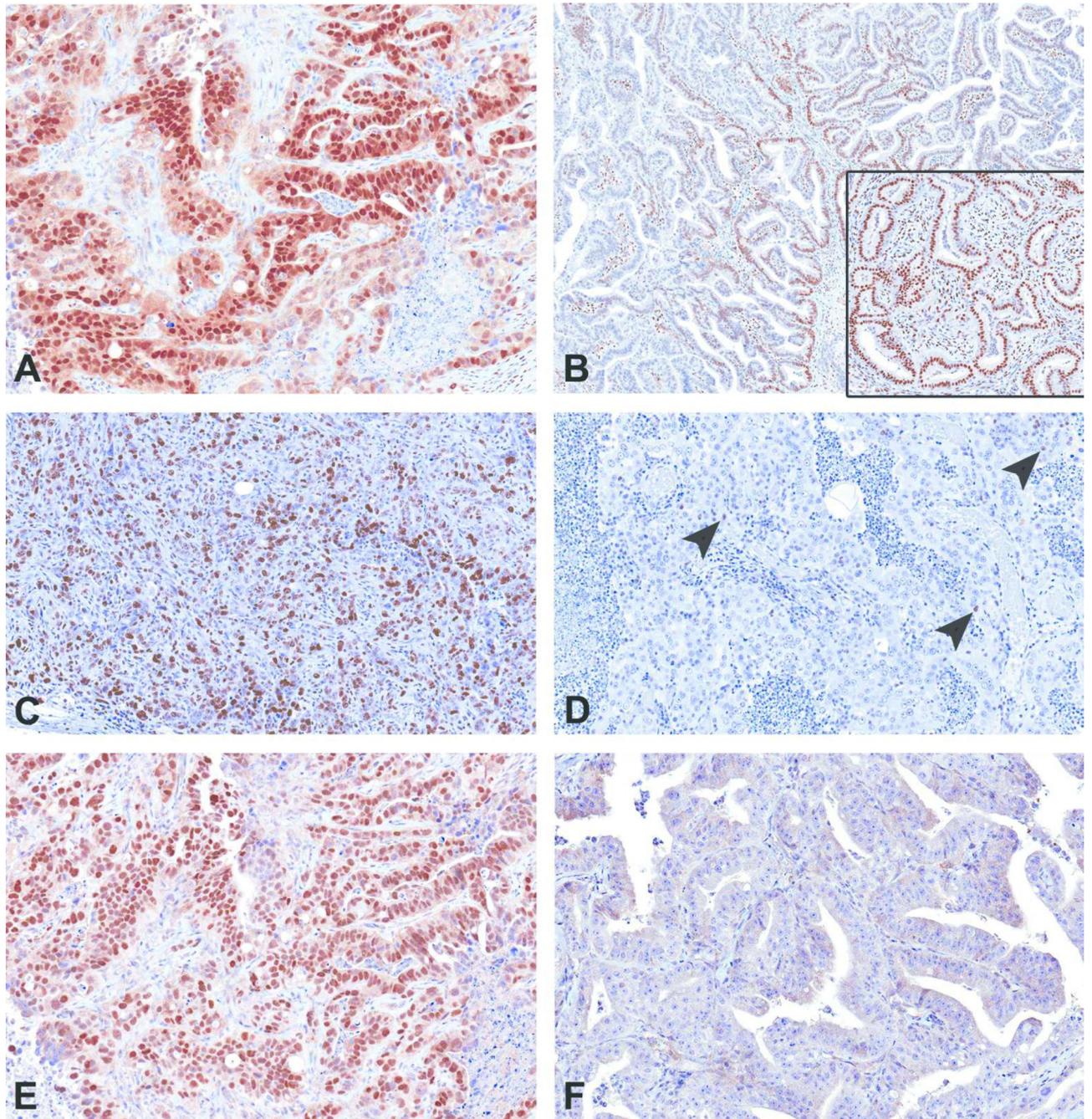
Figure 2.
Immunohistochemical features of uterine carcinomas. (A) All spontaneous and TBBPA-induced uterine adenocarcinomas had diffuse nuclear immunoreactivity to ER α. (B) Two uterine carcinomas from TBBPA-exposed animals had focal nuclear immunoreactivity to PR antibody, and one spontaneous tumor showed diffuse nuclear immunoreactivity (inset). (C) Poorly differentiated uterine carcinomas showed diffuse KI67 expression, whereas well-differentiated tumors (D) had single cell (arrowheads) to no expression of KI67. (E) Uterine

carcinomas from all TBBPA-exposed animals showed strong nuclear immunoreactivity to antibodies to *Tp53*, whereas 4/9 spontaneous tumors were negative (F).
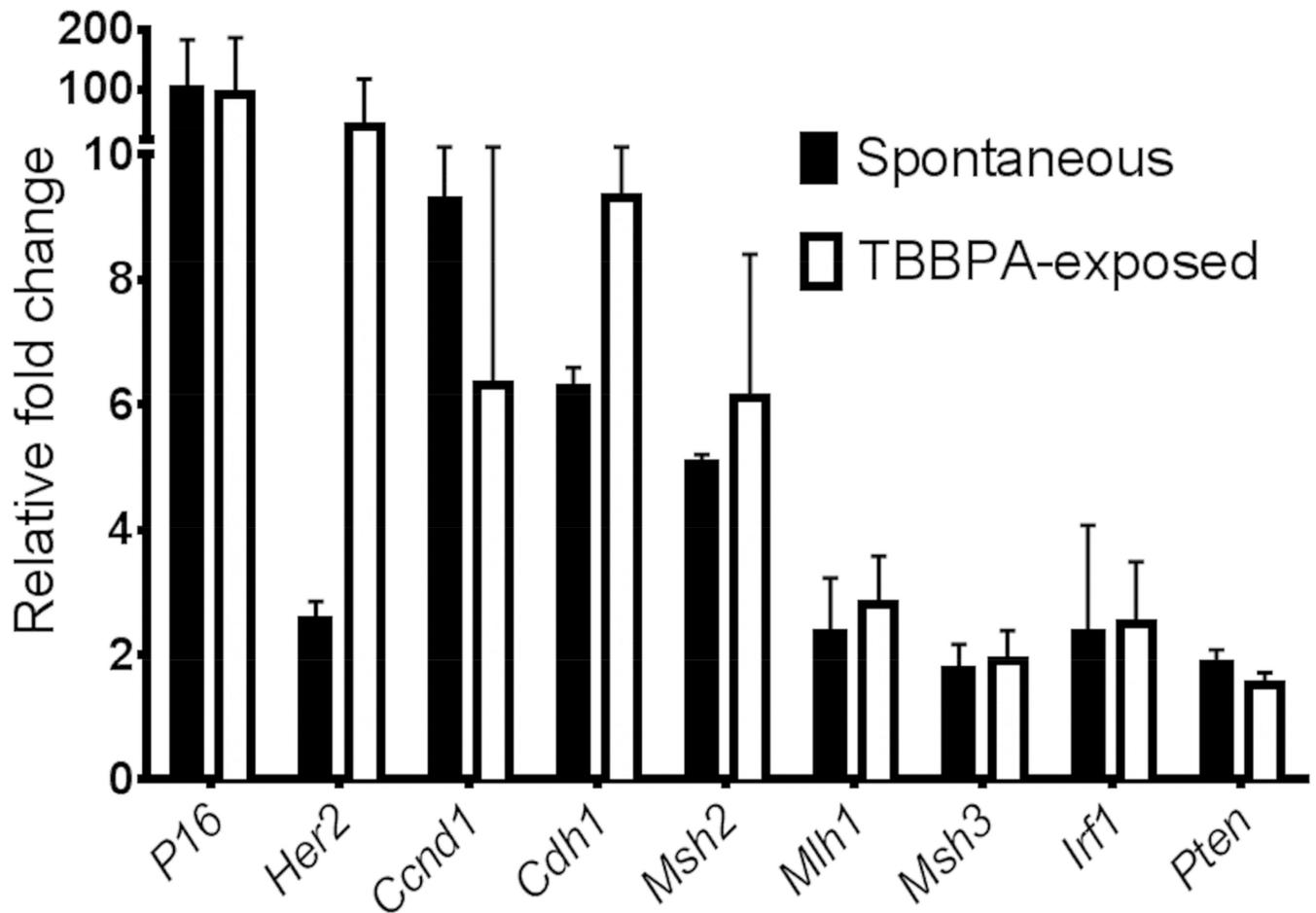
Figure 3.
Real time quantitative PCR gene expression of mediators involved in human endometrial carcinogenesis. Uterine carcinomas in TBBPA-exposed animals averaged a 30 fold overexpression of *Her2* compared to a 2-fold increase in spontaneous uterine carcinomas. Uterine carcinomas from control and TBBPA-exposed animals overexpressed *Ccnd1* and *Cdh1*, although the fold increase in expression was not significantly different between tumors.

Table 1

Clinicopathologic and molecular features of human endometrial carcinoma (EC) subtypes.

	L ow grade Type I EC[a]	High grade Type I EC[a]	Type II EC[a]
Incidence	80–90%	~3%	10–20%
Age	Pre/peri/post-menopausal	Pre/peri/post-menopausal	Post-menopausal
Parity	Nulliparous	Nulliparous	Multiparous
Morphology	Endometrioid	Overlapping Type I & Type II features	Clear/Serous cell
Background	Hyperplastic	Hyperplastic	Atrophic
Invasion	Less invasive	Local invasion	Infiltrative/peritoneal seeding
Behavior	Stable	Aggressive	Aggressive
Prognosis	Favorable	Poor (relative to Low grade Type I)	Poor
ER-α expression	Present	Absent	Absent
PR expression	Present	Absent	Absent
TP53 mutation	10–20%	Higher than Low grade	80–90%

[a]Horn *et al.*, 2007; Liu 2007; McConechy *et al.* 2012; Mittal and Barwick, 1988; Ryan *et al.*, 2005; Zannoni *et al.* 2010.

Table 2

Standardized system of nomenclature and diagnostic criteria (SSNDC) classification of endometrial adenocarcinomas (Dixon *et al* 1999; 2014).

Well-Differentiated Endometrial Adenocarcinomas
Usually cuboidal or columnar neoplastic epithelial cells arranged in well-formed solid nests, cords, papillary or acinar structures within or supported by stroma
Poorly Differentiated Endometrial Adenocarcinomas
Neoplastic epithelia exhibit cellular and nuclear atypia and pleomorphism. Multiple cell layers thick and may pile upon or crowd one another

Table 3

Primers used to amplify hotspot regions of rat *Kras Ctnnb1 Tp53* and *Pten*.

Gene	Exon	Codon	Primer	Strand	Sequence
Kras	1	12–13	*KrasF25849*	Sense	5'-AAAGTACTTGATAATTCTTGTGTGG-3'
			KrasR25489	Antisense	5'-AGAAGTGGGGTTCCTTGCACCG-3'
			KrasR25497	Antisense	5'-GGTTCCTTGCACCGATGGTTCCC-3'
	2	61	*KrasF14399*	Sense	5'-GCTCTGCCTATTTGGACTGGG-3'
			KrasF14444	Sense	5'-CGAGTGCTTGCTGCGACCACTTG-3'
			KrasR13986	Antisense	5'-CAGGAATTCTACATACTTGACAC-3'
Ctnnb1	2	5–80	*CatF272*	Sense	5'-ACATAATCAACAAGCCACCC-3'
			CatF431	Sense	5'-ACTCAGGCAGCATTCTCAGTGCAT-3'
			CatR7 25	Antisense	5'-GGAAGGTAACACAGAGAGTTGCTT-3'
			CatR799	Antisense	5'-ATGTGAGACTCCGTTGCC-3'
Tp53	5	124–184	*p53Ex5OF1366*	Sense	5'-CCTAGTTGGCTTGTCCG-3'
			p53Ex5OR1671	Antisense	5'-AGCAAGAATAAGTCAGAGGC-3'
			p53Ex5IF1382	Sense	5'-CGCTGACCTTTGATTCTTTCTCC-3'
			p53Ex5IR1639	Antisense	5'-GACAACCAGTTCTAAACCCCACAG-3'
	6	185–259	*p53Ex6OF1620*	Sense	5'-TGGGGTTAGAACTGGTTG-3'
			p53Ex6OR1963	Antisense	5'-GAACAAAAACAGGCCGAG-3'
			p53Ex6IF1645	Sense	5'-TCTCCCGGCCTCTGACTTATTC-3'
			p53Ex6IR1927	Antisense	5'-CAGCCCAACCTGGCACAC-3'
	7	260–304	*p53Ex7OF2101*	Sense	5'-AGCTCCAGATAGGACAAG-3'
			p53Ex7OR2434	Antisense	5'-TGGGCAGTGCTATGGAAG-3'
			p53Ex7IF2166	Sense	5'-AGCTTTCTTACTGCCTTGTG-3'
			p53Ex7IR2402	Antisense	5'-TGACTTTGGGGTGAAGCTG-3'
	8	305–329	*p53Ex8OF2333*	Sense	5'-GGAGTGCAAAGAGAGGTG –3'
			p53Ex8R2602	Antisense	5'-TGCGCTCTGACGATAATG-3'
			p53Ex8IF2386	Sense	5'-GCTTCACCCCAAAGTCAC-3'
			p53Ex8IR2549	Antisense	5' - GCGTTTTGTGTCCTAGACTTAG-3'
Pten	1–7	1–260	*RPtenex1F*	Sense	5'-TGACAGCCATCATCAAAGAG-3'

Gene	Exon	Codon	Primer	Strand	Sequence
			R*Ptenex7R*	Antisense	5'-CTTTGAGCATCTTGTTCTGTT-3'
	6–9	173–404	R*Ptenex6F*	Sense	5'-GGAGTAACTATTCCCAGTCAGAG-3'
			R*Ptenex9R*	Antisense	5'-TCAGACTTTTGTAATTTGTGAAT-3'

Table 4

Incidence of epithelial uterine neoplasms in TBBPA-exposed Wistar Han rats in the two-year NTP bioassay.

UTERUS[a]	0mg/kg	250mg/kg	500mg/kg	1000mg/kg
Adenoma (includes multiples)	3 (6%)	2 (4%)	4 (8%)	6 (12%)
Poly-3 test[b]	P=0.103	P=0.489N	P=0.437	P=0.242
Adenocarcinoma (includes multiples)	4 (8%)	10 (20%)	15 (30%)	16 (32%)
Poly-3 test	P=0.002^	P=0.089	P=0.002*	P=0.002*

[a] n = 50 animals examined per group

[b] The p-value in the control column corresponds to the trend test; p-values in the dose columns correspond to pairwise comparisons with the control group.

N indicates a tumor-rate decrease compared to controls.

^ Statistically significant (p < 0.05) based on poly-3 test for linear trend.

* Statistically significant (p < 0.05) based on poly-3 test for pairwise comparisons to the controls.

Table 5

Morphologic and immunohistochemical features of uterine carcinomas from control and TBBPA-exposed WH rats.

	TBBPA-treated	Control
Morphology		
Poorly-differentiated	21/22 (95%)	8/10 (80%)
Well-differentiated	1/22 (5%)	2/10 (20%)
Estrogen receptor-α expression	6/6 (100%)	9/9 (100%)
Progesterone receptor expression	2/6 (33%)	6/9 (67%)
TP53 expression	6/6 [2.3][a]	5/9 [1.0] [a]
KI67 expression	6/6 [2.8] [a]	9/9 [1.7] [a]

[a]Average intensity scores are an average of the severity grade (0 = no immunoreactivity, 1+ = minimal, 2+ = moderate, 3+ = strong immunoreactivity) for each animal divided by the total number of animals in each cohort (n=6 for TBBPA-treated rats; n=9 for control rats).

Table 6

Mutation frequency of Tp53, Ctnnb1, and Kras in uterine carcinomas from control and TBBPA-exposed WH rats.

	TBBPA-treated	**Control**
Tp53 mutation	15/22 (68%)	2/10 (20%)
Ctnnb1 mutation	3/22 (14%)	0/10 (0%)
Kras mutation	1/22 (4.5%)	0/10 (0%)

Table 7

Morphologic and molecular features in uterine carcinomas from humans and TBBPA-exposed WH rats.

	Morphologic		
	Human Type I EC[a]	**Human Type II EC**[a]	**TBBPA-exposed WH rat**[b]
Relative Incidence	80–90%	10–20%	~27% (41/150)
Age	Pre/peri/post-menopausal	Post-menopausal	Late onset
Histology	Endometrioid	Serous/clear cell	Endometrioid
ERa expression	Present	Absent	Present
Background	Hyperplastic	Atrophic	
Differentiation	Low grade	High grade	High > Low grade
Invasion	Minimal	Deep	Deep > Minimal
Behavior	Stable	Progressive	
Prognosis	Favorable	Poor	

	Molecular		
	Human Type I EC[a]	Human Type II EC[a]	TBBPA-exposed WH rat[b]
PTEN inactivation	35–80%	10%	0%
Microsatellite instability	20–45%	0–5%	ND
KRAS mutation	10–30%	0–5%	4.5%
CTNNB1 mutation	20–40%	0–5%	14%
PIK3CA mutation	30%	5%	ND
TP53 mutation	10–20%	80–90%	68%
HER2 overexpression	10–30%	40–80%	100% (30+ fold)
P16 inactivation	10%	40%	0%
CDH1 downregulation	10–20%	60–90%	0%
CCND1 overexpression	2–5%	26–42%	100% (6+ fold)

[a] Horn *et al.*, 2007; Llobet *et al.*, 2009; Lax 2004; Matias-Guiu *et al.*, 2001; Ryan *et al.*, 2005; Liu 2006.

[b] Current study

ND = not determined