ELECTRIC POWER RESEARCH INSTITUTE

COMMENTS OF THE ELECTRIC POWER RESEARCH INSTITUTE ON
IRIS TOXICOLOGICAL REVIEW OF BENZO(A)PRYENE

PREPARED FOR CONSIDERATION BY SCIENCE ADVISORY BOARD CHEMICAL ASSESSMENT ADVISORY COMMITTEE AUGMENTED FOR THE REVIEW OF EPA'S DRAFT BENZO(A)PYRENE ASSESSMENT

April 8th, 2015

The Electric Power Research Institute, Inc. (EPRI) respectfully submits the enclosed comments on the IRIS Toxicological Review of Benzo(a)pyrene to the SAB Chemical Assessment Advisory Committee Augmented for the Review of EPA's Draft BaP Assessment. EPRI thanks the SAB for the opportunity to comment.

EPRI is a nonprofit corporation organized under the laws of the District of Columbia Nonprofit Corporation Act and recognized as a tax exempt organization under Section 501(c)(3) of the U.S. Internal Revenue Code of 1986, as amended, and acts in furtherance of its public benefit mission. EPRI was established in 1972 and has principal offices and laboratories located in Palo Alto, Calif.; Charlotte, N.C.; Knoxville, Tenn.; and Lenox, Mass. EPRI conducts research and development relating to the generation, delivery, and use of electricity for the benefit of the public. An independent, nonprofit organization, EPRI brings together its scientists and engineers as well as experts from academia and industry to help address challenges in electricity, including reliability, efficiency, health, safety, and the environment. EPRI also provides technology, policy and economic analyses to inform long-range research and development planning, as well as supports research in emerging technologies.

EPRI's comments focus on two major issues: (1) lack of real world validation of cancer risks presented in the IRIS document; and (2) differing mutational signatures in human skin tumors versus experimental mouse skin PAH-induced skin tumors. As part of this comment submittal, we will also transmit three published EPRI reports that document PAH concentrations in soil in the United States, and in our comments we recommend that the Agency use these data for the validation exercise.

EPRI has been involved in PAH-related risk assessment research for decades; this focus on environmental and health risk evaluation has been central to EPRI's activities since its inception. These comments on the IRIS assessment reflect EPRI's research activities in that they are technical rather than legal in nature. The comments contained in this letter reflect only EPRI's opinion and expertise and do not necessarily reflect the opinions of those supporting and working with EPRI to conduct collaborative research and development.

EPRI hopes its comments will be valuable to the Committee.

Sincerely,

[redacted]

Anda Ray
Vice President, Environment
And Chief Sustainability Officer

COMMENTS ON IRIS TOXICOLOGICAL REVIEW OF BENZO(A)PYRENE

PREPARED FOR CONSIDERATION BY SCIENCE ADVISORY BOARD CHEMICAL ASSESSMENT ADVISORY COMMITTEE AUGMENTED FOR THE REVIEW OF EPA'S DRAFT BENZO(A)PYRENE ASSESSMENT

Portions Previously Submitted to Docket ID #EPA-HQ-ORD-2011-0391

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April 8, 2015

The Electric Power Research Institute (EPRI) is pleased to provide the following comments on the IRIS Toxicological Review of Benzo(a)pyrene to the Science Advisory Board Chemical Assessment Advisory Committee (CAAC) Augmented for this review. Benzo(a)pyrene (BaP) is a polycyclic aromatic hydrocarbon found in coal tar. EPRI appreciates the opportunity to provide input and hopes that the CAAC will consider our comments.

EPRI is a nonprofit corporation organized under the laws of the District of Columbia Nonprofit Corporation Act and recognized as a tax exempt organization under Section 501(c)(3) of the U.S. Internal Revenue Code of 1986, as amended, and acts in furtherance of its public benefit mission. EPRI was established in 1972 and has principal offices and laboratories located in Palo Alto, California; Charlotte, North Carolina; Knoxville, Tennessee; and Lenox, Massachusetts. EPRI conducts research and development relating to the generation, delivery and use of electricity for the benefit of the public. An independent, nonprofit organization, EPRI brings together its scientists and engineers as well as experts from academia and industry to help address challenges in electricity, including reliability, efficiency, health, safety and the environment. EPRI also provides technology, policy and economic analyses to inform long-range research and development planning, as well as supports research in emerging technologies.

SUMMARY OF KEY COMMENTS

In November 2013, EPRI submitted comments to docket EPA-HQ-ORD-2011-0391 on the public comment draft of the IRIS assessment for benzo(a)pyrene (BaP). Herein, we focus on two specific issues that were discussed in these previously submitted comments as well as in follow-up materials transmitted to EPA in January and February 2014. These issues are summarized as follows:

1. **Lack of real-world validation of cancer risks presented in the IRIS document.** EPRI included this issue in our November 2013 comment document on the public review draft of the IRIS assessment and, in response to a request by IRIS staff, prepared and transmitted an addendum to EPRI's comments on January 31, 2014. This addendum included a detailed calculation of the risk of dermal cancer in coal tar pharmaceutical users. Overall, our calculations show that dermal contact with soil, using data on benzo(a)pyrene toxic equivalents (BaP-TE) for the United States, would account for 26% of all skin cancers on the head, hands, lower legs, and forearms for the entire US population. If stratified by race, EPA's DSF predicts that BaP-TE in background urban soil explains an unrealistic 1,733% of skin cancer in these areas in black Americans. Further, if stratified by body part, EPA's DSF predicts that BaP-TE is responsible for a major portion and often >100% of the actual observed skin cancer in certain areas. Dermal contact with coal tar pharmaceuticals presents a similar situation, with the estimated excess lifetime risk of skin cancer from these products at 0.86. Epidemiological studies of these products have repeatedly shown no increase in skin cancer rates compared to background skin cancer rates in the reference populations. In contrast, EPA's proposed DSF would predict that almost all coal tar pharmaceutical users

should have developed skin cancer. Clearly, when cancer risks presented in the IRIS document are compared to actual levels of skin cancer in the US population, the proposed DSF does not make sense.

2. **Differing mutational signatures in human skin tumors versus experimental mouse skin PAH-induced skin tumors.** EPRI included this issue in our November 2013 comment document on the public comment draft of the IRIS assessment and, in response to a request by IRIS staff, prepared and transmitted an addendum to EPRI's comments on February 25, 2014. The 22-page addendum reviewed 61 papers related to genetic signatures. In the current draft of the IRIS assessment for peer review, EPA does not acknowledge receipt of this addendum, nor of the information contained within it. Our detailed comments below update the original addendum by including additional references in an effort to verify we had consulted all available relevant literature. Overall, the literature search provided evidence that human skin tumors are largely a result of UV light exposure, not of PAH exposure. Specifically, the literature shows that:
    - The majority of human nonmelanoma skin cancers have p53 mutations caused by UV light, typically C to T or CC to TT transitions at specific dipyrimidine sites.
    - In mouse skin photocarcinogenesis experiments, UV-induced mouse skin tumors have the same genetic signature as human basal cell carcinomas and squamous cell carcinomas.
    - On the other hand, mouse skin tumors induced by 7,12-dimethyl benz(a)anthracene (DMBA), BaP, and other PAHs have high rates of mutations in the H-ras proto-oncogene, and have different mutational signatures, most frequently A to T or G to T transversions in specific codons.
    - There is very little evidence that PAH-induced mouse skin tumors contain p53 mutations.
    - There is very little evidence that UV-induced mouse skin tumors, or human skin tumors, contain H-ras mutations.

    Thus, the literature review strongly suggests that human skin tumors have a unique mutational signature attributed to UV exposure, which agrees with the signature of mouse skin tumors induced by UV light, and that mouse skin tumors induced by PAHs contain a distinct and separate mutational signature, which is not seen in human skin tumors. Furthermore, while EPA states in Appendix G of the external review draft that "studies which examine mutational spectra in human skin tumors thought to be related to PAH exposure are not available in the literature", this is because there are no such tumors to examine. Workers exposed occupationally to PAHs do not develop tumors, nor do coal tar pharmaceutical users. Thus, there are no tumors to analyze.

SPECIFIC COMMENTS

1. **<u>Lack of Real-World Validation of Cancer Risks Presented in the IRIS Document</u>**

Risk assessment calculations using the proposed dermal slope factor indicate that high levels of skin cancer caused by potentially carcinogenic PAHs should be seen in the population. The following scenarios provide some insight into the levels of skin cancer that would be expected given the proposed DSF. These calculations were provided in EPRI's comments on the public review draft as well as in an addendum transmitted to IRIS staff in January 2014. They have been updated here to reflect: (a) the increased proposed Dermal Slope Factor (DSF) of 0.006 $(\mu g/day)^{-1}$, (b) the PAH mixture extended analyses performed by Embso-Mattingly, et al. (2014), and (c) the dermal risk assessment equations presented by EPA in Appendix G, Summary of External Peer Review and Public Comments and USEPA's Disposition (EPA, 2014).

Dermal Contact With Soil: The average level of benzo[a]pyrene toxic equivalents (BaP-TE) in soil in urban areas is about 3 mg/kg using the current seven potentially carcinogenic PAHs (EPA, 1993) (Bradley, et al., 1994; EPRI, 2003, 2004, 2008; USGS, 2003) but BaP-TE levels near roads, railways, highways, parking lots, and other places can exceed 3 mg/kg BaP-TE. EPA (2010) has proposed changes to the Relative Potency Factors (RPFs) used to calculate BaP-TE levels and added 18 new PAHs to the list of potentially carcinogenic PAHs. Note that the three EPRI reports referenced above will be transmitted to EPA under separate cover (*Polycyclic Aromatic Hydrocarbons (PAHs) in Surface Soils in Western New York* (Report No. 1005296, October 2003); *Polycyclic Aromatic Hydrocarbons (PAHS) In Surface Soil in Illinois* (Report No. 1011376, December 2004); and *Examination of the Sources of PAHs in Urban Background Soil* (Report No. 1015558, December 2008).

According to Embsbo-Mattingly et al. (2014), BaP-TE for gasoline, diesel, No. 2 fuel oil, crude oil, coal tar, creosote, road pavement, roofing material, & urban sediment will increase by a factor of 10 to 1,000 when EPA's proposed potentially carcinogenic PAHs are added to the BaP-TE calculation. They analyzed the samples with a method that includes 18 potentially carcinogenic PAHs versus EPA's proposed 25. Accordingly, it is assumed for this risk assessment calculation that urban soil contains on average at least ten-fold higher BaP-TE concentrations considering the EPA (2010) proposals, which is 30 mg/kg BaP-TE. The true level of BaP-TE in urban soils including all 25 of the potentially carcinogenic PAHs listed in the 2010 RPF document may be higher.

The lifetime excess cancer risk calculated using the USEPA equation in Appendix G *Summary of External Peer Review and Public Comments and USEPA's Disposition* (EPA, 2014) for soil contact to head, hands, lower legs, and forearms for 70 years, assuming the DSF is correct, is 2.6E-02 for the general population assumed to be exposed to 30 mg/kg BaP-TE. According to the United States Census (2010), 80.7% of the United States population of 309 million in 2010

lived in urban areas. Using today's population of 321 million leads to an exposed population of 259 million.

The lifetime risk of developing nonmelanoma skin cancer at all sites is 2E-01 (Robinson, 2005; Stern, 2010). Half of these skin cancers are present on the head, hands, lower legs and forearms (Scotto, et al., 1983). Thus, the lifetime skin cancer risk for the body parts that EPA has assessed for skin cancer due to BaP-TE in soil is 1E-01. If the proposed DSF is correct, then BaP-TE in soil is the cause of 26% of all skin cancers found on these body parts (2.6E-02/1.0E-01 = 0.26). This prediction does not match the generally recognized fact that ultraviolet radiation in sunlight is the cause of most, if not all, nonmelanoma skin cancer in the general population.

The lifetime risk value of 2E-01 for nonmelanoma skin cancer is often referred to as the risk for the entire population, but the risk value actually refers to the white population of the United States. Almost all nonmelanoma skin cancer surveys have been performed on white Americans, because their skin cancer risk is much higher than the risk for black Americans. Specifically, the nonmelanoma skin cancer rate in black Americans is 68-fold lower than for white Americans, at 3E-03 for all sites and 1.5E-03 for head, hands, lower legs and forearms (Scotto, et al., 1983).

EPA's DSF will be used to estimate skin cancer risks for both white and black Americans. In black Americans, EPA's DSF, using the same EPA equation as above, predicts that BaP-TE in background urban soil explains 1,733% of skin cancer on the head, hands, lower legs, and forearms (2.6E-02/1.5E-03 = 17.33).

Nonmelanoma skin cancer is not prevalent on body parts that are likely to receive exposure to urban soils, such as hands and feet. When EPA's equation and their proposed DSF are used to estimate the lifetime skin cancer risk specifically to hands (3% of skin cancers) or feet (0.1% of skin cancers), the only variable that requires change is the skin surface area exposed. When these calculations are performed, EPA's DSF predicts that BaP-TE is responsible for a major portion and sometimes more than 100% of the actual observed skin cancer.

- Hands, white population 68%
- Hands, black population 4,600%
- Feet, white population 2,500%
- Feet, black population 168,000%

Thus, EPA's DSF predicts that urban background levels of BaP-TE in soil are responsible for more than 100% of the observed nonmelanoma skin cancer at certain anatomical sites and in certain subpopulations. Given that ultraviolet radiation is known to cause most, if not all, nonmelanoma skin cancer, the DSF is simply incorrect and does not predict human risk.

Dermal Contact With Coal Tar Pharmaceuticals: In an externally peer reviewed risk assessment report, ICF (2000) estimated that the average total lifetime exposure of coal tar to patients in the Pittelkow et al. (1981) study of individuals being treated for skin conditions with coal tar pharmaceuticals was 254 grams of absorbed PAHs from coal tar. The average daily dose over the lifetime is 254 grams / (70 years × 365 days/year) = 9.9 mg coal tar/day. The applied dose would be higher, and EPA's proposed DSF is based on applied dose.

The BaP-TE content of coal tar can be taken from Culp et al. (1998), the toxicology study that EPA relies upon for its proposed Oral Slope Factor for benzo[a]pyrene. The BaP-TE for two coal tar samples was 2,696 ppm and 3,965 ppm. The average is 3,331 ppm or 0.003331. The BaP-TE content of the average daily dose of the coal tar pharmaceutical users can be estimated as (9.9 mg coal tar) × (0.003331 BaP-TE/coal tar) = 0.033 mg BaP-TE per day (33 μg BaP-TE per day). Emsbo-Mattingly et al. (2014) analyzed four coal tar products with a method that includes 18 of the EPA's recently proposed list of 25 potentially carcinogenic PAHs (EPA, 2010) and found that the BaP-TE increased 13 to 105-fold compared to the BaP-TE using the historical 7 potentially carcinogenic PAHs (EPA, 1993). Assuming an increase of only 10-fold, this coal tar dose value increases to 330 μg BaP-TE/day.

Assuming the proposed DSF is correct, the estimated lifetime excess lifetime risk of skin cancer for adults only (not including the Age Dependent Adjustment Factors) is 0.86 as noted below. Using the linear equation as used by EPA for estimating risks due to dermal contact with BaP-TE in soil gives a lifetime cancer risk in excess of 1.0.

$$1 - e^{(-330*0.006)} = 0.86$$

The National Psoriasis Foundation states that 2.2% of the population of the United States has psoriasis. If atopic dermatitis/other disorders were included, the fraction would be higher. Assuming the 2.2% of the population have psoriasis or other skin conditions, the population that may use coal tar pharmaceuticals is 7.7 million people. If one assumes that 10% of them use coal tar pharmaceuticals, the exposed population is 770,000 people.

Epidemiological studies of coal tar pharmaceuticals have repeatedly shown no increase in skin cancer rates compared to background skin cancer rates in the reference populations. EPA's proposed DSF would predict that almost all of the thousands of coal tar pharmaceutical users should have developed skin cancer, an outcome that is not observed in these studies.

Dermal Contact With Coal Tar-Based Shampoos: ICF (2000) also derived a dose of 5 μg of coal tar absorbed per day from coal tar shampoo use. Assuming the average BaP-TE content of coal tar from above, 3,331 ppm, the dose of BaP-TE from coal tar shampoo use can be estimated as 5 μg/day × 0.003331 = 0.0167 μg/day. Emsbo-Mattingly et al. (2014) analyzed four coal tar products with a method that includes 18 of the potentially carcinogenic PAHs and found that the BaP-TE increased 13 to 105-fold fold compared to the BaP-TE using the

historical 7 potentially carcinogenic PAHs. Assuming an increase of only 10-fold, this coal tar shampoo dose value increases to 0.167 μg BaP-TE/day.

If the proposed DSF were correct, the estimated excess lifetime cancer risk would be 1.0E-03. Nielsen (1999, see ICF 2000) polled 40,000 households and found that 3,180 had purchased coal tar shampoo (7.95% of people surveyed). Assuming that 7.95% of the United States population uses coal tar shampoos, the exposed population is 27 million people.

2. Differing Mutational Signatures in Human Skin Tumors Versus Experimental Mouse Skin PAH-Induced Skin Tumors

In EPRI's written comments on the public comment draft of the IRIS assessment (December 2013), the following excerpt was included:

"Balmain and Harris (2000) have reviewed the literature and summarized the scientific weight of evidence on carcinogenesis in mouse versus human cells in a paper entitled Carcinogenesis in mouse and human cells: parallels and paradoxes. This paper is not cited in the IRIS document. Balmain and Harris (2000) state that mice are more sensitive to carcinogenic agents than humans. Specifically, they state:

> *"Why should there be such an apparent speeding up of the whole process of carcinogenesis in mice? Although the mutation frequency is thought to be similar in mouse and human cells, cells of rodent origin are in general much easier to transform in culture, either by treatment with exogenous chemicals or by oncogene transfection. It has been speculated that the difference may be due to less efficient DNA repair, poorer control of genetic stability, or altered control of gene expression through processes such as DNA methylation (for review, see ref. 27)."*

Balmain and Harris (2000) present compelling evidence that human skin tumors contain evidence of initiating mutations in the p53 tumor suppressor gene. Similar p53 mutations are seen in the mouse skin tumors caused by UV exposure. However, they are not initiating mutations and are, rather, promoting mutations, because, for instance, p53 null mice do not develop skin tumors unless initiated by chemical agents. The authors present evidence that p53 mutations in the mouse skin induces tumor progression, not tumor initiation. The work of Roop (Greenhalgh et al., 1996) is presented by Balmain and Harris (2000). These researchers found that mice with activated ras genes normally develop skin papillomas, but if they are crossed with p53 null mice, no papillomas form. Thus, the ras mutation is an initiating mutation. Other evidence is presented showing that PAH-initiated mouse skin tumors show mutations induced in the H-ras gene and that the H-ras mutations are initiating. In conclusion, PAH-induced mouse skin tumors have a different Mode of Action than do human skin tumors.

Balmain and Harris (2000) also present a hypothesis that BaP may cause p53 mutations in the lung, so different substances can have different targets in different tissues and even in in different target cells within a tissue (Brown et al., 1998). However, the literature strongly suggests that a required event in mouse skin

tumorigenesis by BaP and other PAHs is an initiating mutation in H-ras. H-ras mutations have not been reported in human skin tumors. Instead, p53 mutations are implicated in the initiation of human skin cancer, which is caused by ultraviolet light.

EPA discusses the fact that BaP-induced mutations have been seen in K-ras, H-ras, and p53 targets in various tumor types in the IRIS document. The only target mentioned in the skin, is H-ras (Chakravati et al., 1995; Wei et al. 1999), which is consistent with the literature summary of Balmain and Harris (2000). EPA discuss p53 as a potential target for BaP in the lung, but there is no mention of p53 being associated with human skin tumors. In fact, there is no discussion of the analysis of genetic defects in human skin tumors at all."

In Appendix G of the peer review draft of the IRIS assessment, the Agency dismisses this comment as follows:

EPA Response: *The review article cited above (Balmain and Harris, 2000) does not discuss PAH-induced skin tumors in mouse skin or human skin. This article reviews the association between benzo[a]pyrene exposure from tobacco smoke and p53 mutations in human lung cancer, the association between sun exposure and p53 mutations in human skin cancer, and the association between dietary aflatoxin B1 exposure and p53 mutations in human liver cancer.*

Benzo[a]pyrene has been shown to be a complete carcinogen in multiple animal species. Skin tumors in mice, rats, rabbits, and guinea pigs have been associated with repeated application of benzo[a]pyrene to skin in the absence of exogenous promoters (Sivak et al., 1997; Grimmer et al., 1984; Habs et al., 1984; Grimmer et al., 1983; Habs et al., 1980; Schmähl et al., 1977; Schmidt et al., 1973; Roe et al., 1970; Poel, 1959). The proposed mutagenic MOA for benzo[a]pyrene involves the bioactivation of benzo[a]pyrene to DNA-reactive metabolites, direct DNA damage by reactive metabolites, formation and fixation of DNA mutations, and clonal expansion of mutated cells. These key events have been observed in animals and humans and by multiple routes of exposure (see Table 1-17 of the Toxicological Review). Benzo[a]pyrene specific skin adducts have been observed in vivo in both benzo[a]pyrene-treated mouse skin and human skin exposed to PAH-mixtures (Godschalk et al., 2001; Rojas et al., 2001; Zhang et al., 1990). In addition, studies of dermal benzo[a]pyrene exposure in mice have shown increased mutations in several gene targets including the tumor suppressor p53 (Ruggeri et al., 1993), the proto-oncogene H-Ras (Wei et al., 1999; Chakravarti et al., 1995) and the lacz transgene (Miller et al., 2000). Studies which examine mutational spectra in human skin tumors thought to be related to PAH exposure are not available in the literature.

The lack of specific H-ras mutational evidence in humans (due to lack of human studies) or suggestive evidence of p53 mutations in UV-induced human skin tumors does not preclude the fact that benzo[a]pyrene can initiate DNA damage via DNA adducts. If not adequately repaired, this damage can form mutations and mutagenesis is a well-established cause of carcinogenesis. Text in the mode of action analysis for carcinogenicity (in Section 1.1.5) has been modified to include studies observing additional target gene mutations (in addition to H-ras) in dermally exposed mice (Miller et al., 2000; Ruggeri et al., 1993).

We would like to refute several elements of EPA's response to our comments. First, EPA states that Balmain and Harris (2000) "does not discuss PAH-induced skin tumors in mouse skin or human skin." In fact, the review paper is dedicated to reporting the genetic signatures of tumors in mice versus humans as noted by selected quotations:

> "Mouse skin tumors initiated with DMBA showed predominantly mutations at the middle adenosine residue of H-ras codon 61 (CAA) which resulted in conversion to thymidine (CTA) and the introduction of an activating missense mutation (49, 50)."
>
> "A number of other model systems and target genes have now been studied and several putative carcinogen-specific mutations have been identified. Space does not allow a comprehensive review of all of this data, but striking examples of the 'smoking gun' concept are the mutations in the p53 tumor suppressor gene in skin tumors induced by UV light..."
>
> "Mutations characteristic of UV exposure have been detected in the p53 gene in human tumors (59), and have been seen in mouse skin tumors induced by UV exposure (60, 61). Different types of mutations are seen in mouse skin tumors induced by repeated exposure to chemical mutagens..."
>
> "Using this 'weight of the evidence' approach, there is a considerable amount of evidence now consistent with the hypotheses that sunlight exposure causes CC -TT tandem mutations in human skin cancer..."

EPA also provided information in response to EPRI's comment about genetic signatures that does not specifically address our comment. EPA stated that benzo[a]pyrene is a complete carcinogen in several animal species, that benzo[a]pyrene can be metabolized, and that benzo[a]pyrene can form DNA adducts. All of these statements are true, but they are irrelevant to the fundamental issue of benzo[a]pyrene's mutational spectrum in the mouse skin compared to the mutational spectrum of human skin cancers.

Out of the large literature on skin cancer genetic signatures, EPA cites four papers in its response to EPRI's comments. Each is discussed below:

Ruggeri et al. (1993)

Ruggeri et al. (1993) studied mutations in skin tumors induced in a complete carcinogenesis protocol with DMBA and BaP. This is one of the few studies that detected mutations in p53 in mouse skin tumors induced by carcinogenic PAHs. However, the authors found that p53 mutations were not present in PAH-induced papillomas, but only in carcinomas, unlike PAH-induced H-ras mutations, which are found in papillomas and carcinomas. The p53 mutations present in the

DMBA-treated mouse skin were of 7 different types and did not indicate any "DMBA signature"; the authors did not implicate the PAH treatment as a causal factor in the p53 mutations.

Wei et al. (1999)

Wei et al. (1999) studied BaP-induced skin cancers in mice and found that 79% of tumors from BaP treated mice had c-Ha-*ras* mutations. The major mutations were G to T transversions in codons 12 and 13. The results of this study are concordant with most of the literature on the genetic signature of PAH-induced mouse skin tumors, which is that PAHs, such as BaP, cause specific G to T transversions in codons 12 and 13 of Ha-*ras* or A to T transversions in codon 61. It is rare for human skin cancers to have these signature H-*ras* mutations.

Chakravarti et al. (1995)

Chakravarti et al. (1995) performed experiments on several PAHs and found: "Dibenzo[a,l]pyrene (DB[a,l]P), DB [a,l]P-11,12-dihydrodiol, anti- DB[a,l] P-11,12-diol-13,14-epoxide, DB[a,l]P-8,9-dihydrodiol, 7,12-dimethylbenz[a]anthracene (DMBA), and 1,2,3,4- tetrahydro-DMBA consistently induced a CAA to CTA mutation in codon 61 of the c-H-ras oncogene." Again, this paper is consistent with the literature, which shows that carcinogenic PAHs cause specific G to T transversions in codons 12 and 13 of Ha-*ra*s or A to T transversions in codon 61 of Ha-*ras.* These mutations are rare in human skin cancers.

Miller et al. (2000)

Miller et al. (2000) studied a transgenic mouse with an *E. coli lacI* transgene as a mutagenesis target. As such, this paper is irrelevant to the issue of comparative genetic signatures of mouse skin and human skin tumors. The paper provides no information on mutations in the Ha-*ras* or p53 genes.

In addition, EPA responded that it could not find any specific evidence of H-*ras* mutations in humans "due to lack of human studies." Actually, there are many studies of human skin tumors summarized in the addendum submitted by EPRI in February 2014. The studies consistently conclude that H-*ras* mutations with a carcinogenic BaP or other PAH signature (i.e., G to T transversions in codons 12 and 13 of Ha-*ras* or A to T transversions in codon 61 of Ha-*r*as) are found in human skin cancers at very low frequency. Instead, C to T or CC to TT transitions are found in certain codons of the p53 gene at high frequency in human skin tumors. Further, while EPA's statement that "studies which examine mutational spectra in human skin tumors thought to be related to PAH exposure are not available in the literature" is true, this is because there are no such tumors to examine. Workers exposed occupationally to PAHs do not develop tumors, nor do coal tar pharmaceutical users. Thus, there are no tumors to analyze.

If EPA's proposed DSF predicted that BaP-TE in urban background soils was the potential cause of a small fraction of human skin cancer, then EPRI would agree with EPA that finding or not finding a specific genetic signature in the human skin tumors that have been genotyped does not rule out a role for BaP-TE in causing human skin cancer. However, as outlined in our comment (1) above, EPA's proposed DSF predicts that BaP-TE in soil in the sole cause of human skin cancer at selected sites, such as hands and feet, and in selected subpopulations, such as black Americans. If BaP-TE, including the other studied PAHs, such as dibenzo[a,h]anthracene and dibenzo[a,l]pyrene, caused more than 100% of skin cancers in these cases, as predicted by EPA's DSF, then a majority of these human skin cancers should contain the PAH-signature mutations, which there is no evidence of.

Below, we present the updated summary of EPRI's addendum submitted in February 2014.

SKIN CANCER GENETIC SIGNATURE SUMMARY

I. Human Nonmelanoma Skin Cancer Genetic Signature

Basal cell carcinomas (BCC) are the most common form of human skin cancer, and squamous cell carcinomas (SCC) are the next most common human skin cancer. Together, the lifetime risk of BCC or SCC in the United States is approximately $2x10^{-1}$ (Robinson, 2005; Stern, 2010). Scientific research overwhelmingly concludes that these skin cancers are caused by ultraviolet light (UV) exposure and, specifically, that these tumors are caused by "signature" mutations in the p53 tumor suppressor gene. The "signature" for UV induced human skin cancer is C to T or CC to TT transitions in the p53 gene at specific dipyrimidine sites.

The scientific weight of evidence agrees with this conclusion (Brash et al., 1991; Shea et al., 1992; Rady et al., 1992; Moles et al., 1993; Daya-Grosjean et al., 1995; Ananthaswamy, 2001; Tsao, 2001; Bolshakov et al. 2003; Giglia-Mari and Sarasin, 2003; Melnikova et al., 2005; Boukamp, 2005; Rebel et al., 2005; Benjamin and Anathaswamy, 2007; El-Deiry, 2007; Benjamin et al., 2008; Rass et al., 2008; Pfeifer, 2010; Rivlin et al., 2011; Loeb et al., 2012; Ikehata, 2013; Ikehata et al., 2013.) No published papers were found that disagree with this conclusion. These papers are discussed individually below, with specific excerpts provided in some cases.

Brash et al. (1991)

"UV light produces distinctive mutations, leaving a "signature" in the DNA. Mutations due to direct absorption of UV light by DNA are predominantly C -- T transitions at dipyrimidine sites, including CC -- TT double-base mutations, in organisms from viral to human (refs. 8-12 and references therein). Because CC - TT base changes are only known to be caused by UV, their presence identifies UV as the mutagen. The appearance of C - T transitions exclusively at dipyrimidines is also unique to UV; chance would dictate that one in four occurs at monopyrimidines."

Shea et al. (1992)

"In summary, we have shown overexpression of p53 protein in a majority of human cutaneous BCCs examined... These findings indicate that mutation of the tumor suppressor gene p53, as a result of chronic UVR exposure, is likely to play an important role in the pathogenesis of BCC of human skin."

Rady et al. (1992)

Rady and colleagues (1992) studied 14 human BCCs and found mutations in the p53 gene in 1 of 14 (50%) of the samples. "All mutations were G:C-A:T transitions, and five (71%) of these mutations were transitions at hot spots with CpG sites, three at codon 248 and two at codon 273. The striking similarity of the type of mutations detected in this study and with the IV mutagenesis studies reported in the literature suggest the hypothesis that UV may act on the p53 gene in a carcinogenic-specific fashion."

Moles et al. (1993)

Moles and colleagues (1993) analyzed 19 human BCCs and 13 human SCCs. 9 of the 19 BCCs (48%) had mutations in the p53 gene and 2 of the 13 SCCs (15%) had mutations in the p53 gene. "Nine of the 11 characterized mutations were single-nucleotide substitutions and, interestingly, seven of these involved CC dimers, where a C was changed into a T or a G (three C-->T transitions and four C-->G transversions). This mutational pattern, added to the fact that all the mutated tumors occurred at sun-exposed body sites, implicates UV light in their genesis."

Daya-Grosjean et al. (1995)

"The analysis of mutation spectra is one of the fundamental approaches used in the elucidation of the mechanisms involved in UV induced mutagenesis. Indeed, the analysis of mutation spectra has proved to be a powerful tool in identifying and demonstrating the role of UV light as the major carcinogen in solar radiation induced human skin cancers due to the unique and characteristic patterns of mutations created in numerous targets."

Ananthaswamy (2001)

"Recent studies indicate that genetic alterations in the p53 tumor suppressor gene play an important role in the development of skin cancer." "Several studies have shown that UV induces unique types of p53 mutations in skin cancers at a high frequency that are not commonly found in other types of human cancer. Analogous to human skin cancers, skin cancers induced in laboratory mice by UV radiation also display UV signature p53 mutations at a high frequency."

Bolshakov et al. (2003)

"p53 mutations were detected in 33 of 50 aggressive BCCs (66%), 37 of 98 nonaggressive BCCs (38%), 28 of 80 aggressive SCCs (35%), 28 of 56 nonaggressive SCCs (50%), and 3 of 29 samples of sun-exposed skin (10%). About 71% of the p53 mutations detected in aggressive and nonaggressive BCCs and SCCs were UV signature mutations. The frequency of CC to TT mutations in aggressive (36%) and nonaggressive SCCs (39%) was 2-fold higher than in aggressive (18%) and nonaggressive (14%) BCCs."

Melnikova et al. (2005)

"UVB radiation induces primarily cyclobutane-type pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts [9–11]. Both "lesions" are formed exclusively in runs of tandemly located pyrimidine residues, which are often "hot spots" for UV-induced DNA damage and mutation [12–14]. These unique lesions in the DNA give rise to unique mutations. That is, UV radiation induces predominantly C→T and CC→TT transitions at the dipyrimidine sequences, which are now recognized as the hallmark of UV-induced mutagenesis [13]." "About 71% of the p53 mutations detected in aggressive and nonaggressive BCCs and SCCs were UV signature mutations. " "Other studies have shown that most human and mouse skin cancers induced by UV radiation display p53 gene mutations at a high frequency [101,112–117]."

Benjamin and Anathaswamy (2007)

"The p53 tumor suppressor gene and gene product are among the most diverse and complex molecules involved in cellular functions. Genetic alterations within the p53 gene have been shown to have a direct correlation with cancer development and have been shown to occur in nearly 50% of all cancers. p53 mutations are particularly common in skin cancers and UV irradiation has been shown to be a primary cause of specific 'signature' mutations that can result in oncogenic transformation."

"In UV-induced skin cancer, the frequency of C to T transitions is especially frequent at the trinucleotide sequence 5′-PyCG in the p53 gene (Setlow and Carrier, 1966). There are several 'hot spot' mutation sites within the p53 gene. Data collected from Pfeifer et al. show that of the most commonly mutated sites in p53 five are mutated dipyrimidine in the sequence context 5′-CCG or 5′-TCG (codons 196, 213, 245, 248, and 282)."

"Mutations in ras oncogenes do not appear to be as important as mutations in the p53 tumor suppressor gene in skin cancer development."

"… the presence of UV signature C→T and CC→TT mutations in the *p53* gene in human and experimental mouse skin cancers has been well documented (Pierceall *et al.*, 1991b; Kress *et al.*, 1992; Rady *et al.*, 1992; Dumaz *et al.*, 1993; Kanjilal *et al.*, 1993; Sato *et al.*, 1993; Ziegler *et al.*,

1993; Greenblatt *et al.*, 1994; van der Riet *et al.*, 1994; Stern *et al.*, 2002; Bolshakov *et al.*, 2003)."

"A number of investigators have detected *p53* gene mutations in a large proportion of human squamous cell carcinomas and basal cell carcinomas (Pierceall *et al.*, 1991a; Pierceall *et al.*, 1991b; Rady *et al.*, 1992; Dumaz *et al.*, 1993; Moles *et al.*, 1993; Sato *et al.*, 1993; Ziegler *et al.*, 1993; Greenblatt *et al.*, 1994; van der Riet *et al.*, 1994; Ziegler *et al.*, 1994; Stern *et al.*, 2002; Bolshakov *et al.*, 2003). Initial studies by Brash and co-workers (Brash *et al.*, 1991) revealed *p53* mutation in 58% of human SCC. Later studies by Ziegler et al. (1994), and Rady et al. (1992) have demonstrated *p53* mutations in human BCCs at 56% and 50% frequencies, respectively. Interestingly, Ziegler et al. (1994) found that 45% of human BCCs contained a second point mutation on the other *p53* allele. More recently, Bolshakov et al. (2003) analyzed 342 tissues from patients with aggressive and nonaggressive BCCs and SCCs for p53 mutations. p53 mutations were detected in 66% BCCs, 38% of nonaggressive BCCs, 35% of aggressive SCCs, 50% of nonaggressive SCCs, and 10% of samples of sun-exposed skin. About 71% of the p53 mutations detected in aggressive and nonaggressive BCCs and SCCs were UV signature mutations (Bolshakov *et al.*, 2003)."

El-Deiry (2007)

"UV exposure causes characteristic cyclobutane pyrimidine dimers and 6-4 photoproducts leading to "signature mutations," such as CC to TT, in hot spots within the p53 gene."

Benjamin et al. (2008)

"p53 mutations are particularly common in skin cancers and UV irradiation has been shown to be a primary cause of specific 'signature' mutations that can result in oncogenic transformation. There are certain 'hot-spots' in the p53 gene where mutations are commonly found that result in a mutated dipyrimidine site."

"Analysis of mutations in p53 gene has established an unequivocal connection between UV exposure, DNA damage and skin carcinogenesis. UVB and UVC radiation induces unique types of DNA damage, producing cyclobutane-type pyrimidine dimers (CPD) and pyrimidine (6-4) or pyrimidone (6-4) photoproducts."

Giglia-Mari and Sarasin (2003)

"The analysis of p53 mutations in all skin cancers analyzed from DNA repair-proficient patients shows that in vivo in normal human individuals the majority of mutations correspond to C to T transitions almost exclusively at dipyrimidine sites known to be hotspots of DNA lesions following UV-exposure."

Boukamp (2005)

“Which are the genetic lesions that characterize skin carcinoma development and progression? The most prominent and the best studied aberration in skin cancers is the mutation of the p53 tumor suppressor gene.”

“Most importantly, many p53 mutations are C to T transitions with a high frequency of CC to TT double base changes, thus being indicative of UV-radiation-induced mutations (21, 22).”

Rass et al. (2008)

“In most SCC development, UV-signature mutations in the p53 tumor suppressor gene are the most common event, as precancerous lesions ~80% and SCCs >90% UV-specific p53 mutations.”

“They can be identified as UV specific C to T transitions at dipyrimidine sites or CC to TT base changes…”

“Thus, UV-fingerprint mutations can be abundantly detected in the well characterized and pathogenically important tumor suppressor gene p53 from squamous [17] and basal cell carcinoma [23] of human skin. “

Loeb et al (2012)

“Epidemiology studies have implicated Tp53 mutations triggered by UV exposure, and human papilloma virus (HPV) infection to be significant causes of non-melanoma skin cancer.”

“The importance of UV-irradiation in the development of cutaneous squamous cell carcinoma (SCC) is well established, and studies of premalignant actinic keratosis (AK) lesions and experimental skin cancers in mice suggest that a key event in the development of skin SCC is the acquisition by epidermal keratinocytes of UV-induced Tp53 gene mutations, characteristically (C to T or CC to TT) transitions at dipyrimidine sites [6,7,8].”

Pfeifer (2010)

“These studies of TP53 mutations have found UVB-specific mutations, C-to-T transitions at dipyrimidine sequences and CC-to-TT tandem mutations as hallmarks of sunlight exposure leading to non-melanoma skin cancers [6].”

Rivlin et al. (2011)

“Exposure to sunlight and UV radiation has also been implicated in genetic transitions in *TP53* in the skin, leading to cancer development. About 50% of skin cancers exhibit *TP53* mutations that are characterized by specific C to T and CC to TT transitions, a signature of UVB-induced mutagenesis [48, 49].

Ikehata (2013)

"The solar-UV signatures were detected in the transgenes of mouse skin exposed to UVB, sunlight and UVA in studies with *lacZ*-transgenic mice[4] as well as in the p53 genes of mouse skin cancers induced experimentally by those UVR sources[5-7]. Those solar-UV-signature mutations detected in mice were found to prefer TCG to CCG for their target sites[4]. The p53 mutations in human nonmelanoma skin cancers (NMSCs) also show the UV signature, which strongly supports the idea that NMSCs are caused by solar UVR exposure, and include solar UV-signature mutations[2,8]."

II. Ultraviolet Light Induced Mouse Skin Cancer Genetic Signature

In mouse skin photocarcinogenesis experiments, UV-induced mouse skin tumors have the same genetic signature as human BCCs and SCCs. The "signature" for UV induced mouse skin cancer is also a C to T or a CC to TT transition in the p53 gene at dipyrimidine sites (Brash et al., 1991; Kanjilal et al.,1993; Nataraji et al., 1995; Ananthaswamy et al., 1997; Ananthaswamy et al. 1998; Dumaz et al., 1997; Ananthaswamy et al., 1999; Jiang et al., 1999; Ananthaswamy, 2001; Bolshakov et al. 2003; Rebel et al., 2005; Melnikova et al., 2005; Benjamin and Anathaswamy, 2007, Schwarz et al., 2012).

For instance, Dumaz et al. (1997) reported: "The observed mutation frequency after chronic UV-B radiation in the p53 gene ranged from 54% (SKH-HRA) to 73% (SKH-HR1) among the 160 tumors analyzed. Over 95% of the mutations were found at dipyrimidine sites located in the non-transcribed strand, the majority were C-T transitions and 5% were CC-TT tandem double mutations."

"It has already been shown that the p53 mutation spectrum in human skin cancers is significantly different from those observed in other human cancers but very similar to those observed in UV-treated target genes in model systems (2, 3). In human skin carcinomas, p53 mutations are mainly C-T transitions located on dipyrimidine sites that are specific UV targets…"

Ananthaswamy et al. (1998) stated: "Several studies have shown that both human and UVR-induced mouse skin cancers harbor unique mutations (C + T and CC -+ TT transitions) at dipyrimidine sites that are not commonly found in other types of human cancers (1-9)."

Kanjilal et al. (1993) published a similar conclusion: "Similar UV-specific mutations in the p53 gene have also been detected in human (2, 10, 11) and murine (12) skin tumors."

Nataraji et al. (1995) reported: "…our studies have demonstrated p53 mutation at an unprecedented 100% frequency in UV-induced C3H mouse skin tumors.[21]"

Jiang et al. (1999) also reported: "In UV-associated skin carcinogenesis in both humans and mice, mutations in p53 are present with very high frequency (Brash et al., 1991; Kanjilal et al., 1993)."

Similarly, Benjamin and Anathaswamy (2007) reported: "… the presence of UV signature C→T and CC→TT mutations in the p53 gene in human and experimental mouse skin cancers has been well documented (Pierceall et al., 1991b; Kress et al., 1992; Rady et al., 1992; Dumaz et al., 1993; Kanjilal et al., 1993; Sato et al., 1993; Ziegler et al., 1993; Greenblatt et al., 1994; van der Riet et al., 1994; Stern et al., 2002; Bolshakov et al., 2003)."

Schwarz et al. (2012) stated: "UV-induced human SCC show footprint mutations in the tumor suppressor gene TP53 which are also observed in UV-induced mouse SCC."

III. Conclusion Regarding Ultraviolet Light Induced Skin Cancers

The most common skin cancers worldwide, BCCs and SCCs, are caused by exposure to ultraviolet radiation in sunlight. The UV radiation specifically causes C to T and CC to TT transition mutations in the p53 tumor suppressor gene. These specific mutations are also seen in mouse skin photocarcinogenesis models.

IV. PAH Induced Mouse Skin Cancer Genetic Signature

On the other hand, mouse skin tumors induced by 7,12-dimethyl benz(a)anthracene (DMBA), benzo(a)pyrene (BaP), and other PAHs have high rates of mutations in the H-*ras* proto-oncogene (Balmain and Pragnell, 1983; Balmain et al., 1984; Quintanilla et al., 1986; Barbacid, 1987; Bailleul et al., 1989; Fujiki et al., 1989; Brown et al., 1990; Buchmann et al., 1991; Nelson et al., 1992; Kemp et al., 1993; Colapietro et al., 1993; DiGiovanni et al., 1993; Chakravarti et al., 1995; Ross and Nesnow, 1999; Chakravarti et al., 1998; Melendez-Colon et al., 1999; Wei et al., 1999; Ise et al., 2000; Chakravarti et al., 2000; Pazzaglia et al., 2001; Khan et al., 2005; Chakravarti et al., 2007; To et al., 2013.)

In addition to having a specific gene as the target in PAH-induced skin tumors, these PAH-induced skin tumors have specific mutational "signatures" that differ from the signatures caused by different chemical carcinogens. Specifically, the PAH skin cancer "signature" is an A to T transversion in codon 61 of H-*ras*, a G to T transversion in codon 12 of H-*ras*, or a G to T transversion in codon 13 of H-*ras*.

The predominant mutation in skin tumors induced by DMBA is an A to T transversion in codon 61 of H-*ras* (Quintanilla et al., 1986; Bailleul et al., 1989; Nelson et al., 1992; Kemp et al., 1993; Colapietro et al., 1993; Chakravarti et al.,1995; Ross and Nesnow, 1999; Melendez-Colon et al., 1999; Pazzaglia et al., 2001).

The predominant mutations in skin tumors induced by BaP are A to T transversions in codon 61 of H-*ras* (Buchmann et al., 1991; Colapietro et al., 1993; Chakravarti et al., 1995; Melendez-Colon et al., 1999) and G to T transversions in codon 12 or 13 of H-*ras* (Bailleul et al., 1989; Colapietro et al., 1993; Chakravarti et al., 1995; Melendez-Colon et al., 1999; Wei et al., 1999).

DMBA: A to T Transversions in Codon 61

Quintanilla et al. (1986) found that over 90% of mouse skin tumors induced by DMBA have a specific A to T transversion at the second nucleotide of codon 61 of the Harvey ras (Ha-ras) gene.

Barbacid (1987) reported that in mice "H-ras- l oncogenes have been found to be reproducibly activated in 90% of skin papillomas and carcinomas of Sencar mice initiated by DMBA or dibenz(c,h)acridine (DBACR) treatment and followed by promotion with TPA (211 -213)." "Induction of skin carcinomas in mice by DMBA and phorbol esters involves the specific activation of H-ras- l oncogenes by A - T transitions in the second base of codon 61 (212, 213)."

Bailleul et al. (1989) reported: "Our previous experiments on the role of oncogenes in mouse skin carcinogenesis showed that a member of this family (Ha-ras) was reproducibly activated by point mutations in both papillomas and carcinomas initiated with dimethylbenz[a]anthracene (DMBA) (6-8)." "An AT--TA transversion mutation is seen in a very high proportion of the tumors initiated with DMBA."

Brown et al. (1990) studied the effects of DMBA on mouse skin from the Beatson Institute and found that 45 out of 48 (94%) papillomas and 10 out of 13 (76%) showed A to T transversions at codon 61 in Ha-*ras*. The authors concluded: "Results presented demonstrate the carcinogen-specific nature of these ras mutations. For each initiating agent, a distinct spectrum of mutations is observed."

Nelson et al. (1992) tested DMBA-induced mouse skin tumors and found: "An activated Ha-ras oncogene has been consistently found in chemically initiated benign and malignant mouse skin tumors, and an activated ras oncogene has been shown to initiate the process of mouse skin carcinogenesis." "Molecular analysis of these tumors has revealed the presence of an A – T transversion mutation at the 61st codon position of the Ha-ras gene."

Kemp et al. (1993) found the same result in DMBA-induced mouse skin tumors. "Most tumors from all groups had activating mutations in the H-ras gene." "Eight out of 9 papillomas and 3 out of 4 carcinomas from the null mice did, in fact, have the A-T transversion mutation in codon 61 of the H-ras gene, as did the majority of tumors from the heterozygous mice (14 out of 15) and wild-type mice (5 out of 5) (Figure 3)." "Previous work from this and other laboratories has shown that more than 90% of DMBA/TPA-induced papillomas and carcinomas contain the same point mutation in codon 61 of the H-ras gene (Quintanilla et al., 1986; Bizub et al., 1986)."

Colapietro et al. (1993) studied BaP, but they summarized the DMBA literature: "Various chemical carcinogens display a differential reactivity with specific base residues in DNA. For example, 7,12-dimethylbenz[a]anthracene (DMBA) is a prototypical mouse skin initiating carcinogen which preferentially covalently binds to adenine residues (22) and exhibits a high frequency (> 90%) of A—T transversions in the 61st codon of the Ha-ras gene, as reported by

Balmain et al. (23). Furthermore, these mutations can be detected in DMBA-initiated mouse skin before the appearance of tumors (24)."

Chakravarti et al. (1995) performed experiments on several PAHs including DMBA and found: "Dibenzo[a,l]pyrene (DB[a,l]P), DB [a,l]P-11,12-dihydrodiol, anti- DB[a,l] P-11,12-diol-13,14-epoxide, DB[a,l]P-8,9-dihydrodiol, 7,12-dimethylbenz[a]anthracene (DMBA), and 1,2,3,4-tetrahydro-DMBA consistently induced a CAA to CTA mutation in codon 61 of the c-H-ras oncogene"

Ross and Nesnow (1999) summarized the literature on and concluded: "The analysis of Ha-ras mutations is dominated by studies using DMBA (Table 2). In these studies, codon 61 exhibits a mutational hot spot with the second position of CAA, a target, resulting in A to T transversions (CTA)."

Melendez-Colon et al. (1999) performed mechanistic studies with mouse skin tumors induced by DMBA, BaP and dibenzo[a,l]pyrene (DB[a,l]P) and reported: "Nucleotide transversions within codons 12 (G to T) or 61 (A to T) of cellular *H-ras* have frequently been found as activating mutations upon exposure to carcinogenic PAHs such as B[a]P (58), DMBA (5,59,60) or DB[a,/]P (45). Exposure to B[a]P and DB[a,/]P mainly results in the induction of G to T and A to T transversions, respectively, whereas DMBA treatment caused both kinds of mutations in these two codons (5,45,58,59)."

Ise et al. (2000) induced skin tumors in mouse skin using DMBA. 17 out of 17 skin tumors examined in wild time mice had mutations in codon 61 of the H-*ras* gene. H-*ras* deficient mice has greatly lower rates of tumor formation.

Pazzaglia et al. (2001) studied DMBA-induced skin cancer in carcinogenesis-susceptible and carcinogenesis-resistant mice and found: "The data showed that a A^{182} to T transversion in the c-Ha-ras gene was present in 100% and 81% of the skin tumors developed in Car-S and Car-R mice, respectively, after DMBA initiation and TPA promotion, suggesting that differences in genetic susceptibility can influence the frequency of c-Hras mutations in the skin tumors produced."

"In the classic two-stage murine skin carcinogenesis model, initiation of tumorigenesis by 7,12- dimethylbenz[a]anthracene (DMBA) induces a specific point mutation in codon 61 (A^{182} --- T) of the c- Ha-ras gene, causing a Gln to Leu substitution in the ras protein [2]. In mouse strains or lines sensitive to skin tumor development, repeated applications of a tumor promoter after initiation lead to the appearance of papillomas, some of which can progress to malignant carcinomas. Papillomas are considered clonal expansions of the cells initiated by activation of the c-Ha-ras gene. Nearly all (90±100%) the papillomas produced by DMBA initiation and 12-Otetradecanoylphorbol-13-acetate (TPA) promotion showed this restricted mutational spectrum [2±4]."

BaP: A to T Transversions in Codon 61

Buchmann et al. (1991) studied DMBA and BaP-induced mouse skin tumors and found that 2 of 2 carcinomas induced by repeated B[a]P treatment contained the A — T transversion in the 61st codon of H-*ras.*

Colapietro et al. (1993) studied BaP-induced mouse skin cancer and found: "We have demonstrated that 90% of benzo[a]pyrene initiated/TPA-promoted early emergence papillomas contain a mutation in the Ha-*ras* gene. Furthermore, 70% of these papillomas demonstrate a G—T mutation in either the 12th or 13th codons and 20% demonstrate an A—T mutation in the 61st codon. Since BPDE preferentially binds to guanine residues in DNA (27,28) and to a lesser extent adenine residues (29,30), the mutations we observed in the Ha-*ras* gene are consistent with the known binding affinity of the ultimate carcinogenic species of B[a]P for specific bases."

"In summary, we have demonstrated a 90% incidence of the Ha-ras mutation in B[a]P-initiated/TPA-promoted early emergence papillomas: 20% contained a GGA —GTA transversion in the 12th codon, 50% contained a GGC—GTC transversion in the 13th codon and 20% contained a CAA—CTA transversion in the 61st codon."

Chakravarti et al. (1995) performed experiments on several PAHs including BaP and found: "Benzo[a]pyrene induced a GGC to GTC mutation in codon 13 in 54% of tumors and a CAA to CTA mutation in codon 61 in 15%."

Melendez-Colon et al. (1999) performed mechanistic studies with mouse skin tumors induced by several PAHs, including BaP and reported: "Exposure to B[a]P and DB[a,/]P mainly results in the induction of G to T and A to T transversions, respectively, whereas DMBA treatment caused both kinds of mutations in these two codons (5,45,58,59)." (Note added: The authors are referring to codons 12 and 61 of H-*ras*.)

Anti-benzo[c]phenanthrene, 3,4-diol-epoxide: A to T Transversions in Codon 61

Ronai et al. (1994) studied mouse skin tumors induced by anti-benzo[c]phenanthrene, 3,4-diol-epoxide in CD-1 mice. Codon 61 mutations of the Ha-*ras* gene were seen in 41 of 65 (63%) of the tumors. 31 of the 41 codon 61 mutations (90%) were A to T transversions.

DB[a,l]P: A to T Transversions in Codon 61

Chakravarti et al. (1998) studied DB[a,l]P-induced skin tumors in mice and found: "Papillomas induced by the most carcinogenic environmental polycyclic aromatic hydrocarbon (PAH), dibenzo[a,l]pyrene (DB[a,l]P), in SENCAR mouse skin contain a specific H-ras codon 61 (CAA to CTA) mutation."

Khan et al. (2005) studied DB[a,l]P-induced skin tumors in mice and found: "Topical application of dibenzo[a,l]pyrene (DB[a,l]P) to the dorsal skin of SENCAR mice induces codon 61 (CAA^{Gln} to CTA^{Leu}) mutations in the Harvey (H)-ras gene within 12 h after treatment.

Chakravarti et al. (2000) further studied DB[a,l]P and reported: "Mice treated on the dorsal skin with the potent polycyclic aromatic hydrocarbon (PAH) carcinogen dibenzo[a,l]pyrene (DB[a,l]P) form papillomas carrying the H-ras codon 61 (CAA to CTA) mutations."

BaP: G to T Transversions in Codons 12 or 13

Bailleul et al. (1989) studied BaP-induced mouse skin tumors and reported G to T transversions in codons 12 and 13 of H-*ras*.

Colapietro et al. (1993) studied BaP-induced mouse skin cancer and found: "We have demonstrated that 90% of benzo[a]pyrene initiated/TPA-promoted early emergence papillomas contain a mutation in the Ha-*ras* gene. Furthermore, 70% of these papillomas demonstrate a G—T mutation in either the 12th or 13th codons…"

Chakravarti et al. (1995) performed experiments on several PAHs including BaP and found: "Benzo[a]pyrene induced a GGC to GTC mutation in codon 13 in 54% of tumors and a CAA to CTA mutation in codon 61 in 15%."

Melendez-Colon et al. (1999) reported G to T transversions in codon 12 of H-*ras* as noted above.

Wei et al. (1999) studied 154 BaP-induced skin cancers in mice and found that 79% of tumors from BaP treated mice had c-Ha-*ras* mutations. The major mutations were G to T transversions in codons 12, 13 or 61. The most prevalent mutation was a G to T transversion in codon 13.

V. Evidence that PAHs May Induce p53 Mutations

There is scant evidence that PAH-induced mouse skin tumors contain p53 mutations. As noted below, low levels of p53 mutations in PAH-treated mouse skin have been reported in a handful of studies, but the researchers do not implicate them as causal events in mouse skin tumorigenesis. Burns et al. (1991) conclude that the p53 mutations were spontaneous in origin, as noted below.

Ruggeri et al. (1991) studied p53 mutations in several cell lines derived from DMBA-induced mouse skin tumors. No primary tumors were analyzed. Several cell lines showed p53 mutations, but they were not of the type seen in human skin tumors (C to T transitions). They were also not the type of mutation seen with PAH-induced mouse skin tumors (A to T transversions or G to T transversions). Instead, they were G to C transversions. The authors concluded that they were not the proximate cause of the skin cancer: "In this study, we present data which suggest that alterations in the p53 tumor suppressor gene are absent or rare in papillomas and early well-differentiated carcinomas and are later events in the process of murine skin tumor progression."

Ruggeri et al. (1993) studied mutations in skin tumors induced in a complete carcinogenesis protocol with DMBA and BaP. They found that p53 mutations were not present in PAH-induced papillomas, but only in carcinomas, unlike PAH-induced H-ras mutations, which are found in papillomas and carcinomas. The p53 mutations present in the DMBA-treated mouse skin were of 7 different types and did not indicate any "DMBA signature." The authors did not implicate the PAH treatment as a causal factor in the p53 mutations.

"The results of the present study of p53 mutations in murine B[a]P-induced skin tumors reveal a 33% frequency of missense mutations predominantly G to T transversions in exons 7 and 8 of the p53 gene…" "In contrast, papillomas induced by this protocol were immunohistochemically negative for p53 nuclear staining, and only a single p53 mutation was found by DNA sequence analysis."

"Previous studies in our laboratory (22) and others (31) in the DMBA/PMA-induced model of murine skin tumorigenesis have demonstrated that alterations in the p53 gene are a later event in tumor development-i.e., found in squamous cell carcinomas and more advanced tumors, but absent in papillomas and hyperplastic lesions. Similarly, in the complete carcinogenesis protocols of B[a]P-induced and DMBA-induced skin tumorigenesis, p53 alterations appear to be a rare event in papillomas (35-50 weeks treatment) and predominate in carcinomas (50 weeks treatment). In contrast to our previous studies (22), however, there was no correlation in B[a]P-induced and DMBA-induced carcinomas between the frequency of p53 mutations and the histopathological grade of the carcinomas examined (Table 2)."

"It remains to be explained how initial exposure of mouse skin to a carcinogen results in detectable p53 mutations predominantly in carcinomas and not in benign lesions, or only at a low frequency in the latter."

Burns, et al. (1991) studied five carcinomas and two cell lines from DMBA-induced mouse skin. They found the DMBA "signature" A to T transversion in codon 61 of H-ras. In addition, they found p53 mutations, but the p53 mutations present in the DMBA-treated mouse skin were of 5 different types and did not indicate any "DMBA signature" despite the fact that there was one A to T transversion. Burns et al. (1991) concluded that the p53 mutations were not initiating events and were not likely caused by the DMBA treatment. Instead, they were spontaneous mutations, as noted below.

"…loss of normal p53 function is an intermediate event in mouse skin tumor progression, occurring somewhere between benign papilloma formation and malignant transformation to squamous carcinoma."

"Carcinomas SB136, SB142, MSC12.5 and MSC12.12 were all initiated with a single dose of DMBA followed by multiple treatments with the non-mutagenic promoting agent TPA. These four carcinomas all have an A:T to T:A transversion at codon 61 of H-ras, consistent with the known

mutagenic specificity of DMBA. However the p53 mutations in these four tumors comprise three frameshifts and a G: C to A: T transition. This would strongly suggest that the H-ras mutation represented the initiating event in these tumors and that the p53 mutations, which were probably spontaneous in origin, occurred at a later stage."

To et al. (2013) stated: "...topical application of a single dose of 7,12-dimethylbenz(a)anthracene (DMBA) to dorsal skin followed by promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) results in the development of skin tumors, the majority of which harbor a codon 61 activating mutation in the *Hras* gene.[2]" "Chemically induced tumors in mouse lung and skin show complete specificity for mutations involving *Kras* and *Hras*, respectively[2, 3].

VI. Evidence that UV-Induced Mouse Skin Cancer May Contain H-*ras* Mutations

Only two papers were found that mentioned the presence of H-ras mutations in UV-induced mouse skin tumors. Khan et al. (1996) studied photocarcinogenesis in hairless mice. These researchers found low levels of H-ras mutations in tumors from UV-treated mice, but concluded that they were rare and not causal: "With the exception of the low incidence of Ha-ras oncogene mutations at codon 12 in SKH-1 hairless mouse skin tumors induced by UVB, the striking absence of mutations in the Ha-, Ki-, and N-ras oncogenes in UVB-induced mouse skin tumors suggests that ras oncogene mutations are rare and thus are not an initiating event in photocarcinogenesis."

Sutter et al. (1993) analyzed 39 tumors induced by chronic UV radiation in four mouse strains. Only 1 of 30 tumors (3%) demonstrated a mutation in the H-ras gene, and it was a G to A transition in codon 12. There were no mutations in codons 13 or 61. The authors state: "First, our results confirm the existing evidence that UV-induced mutations in *ras* genes of human and murine epidermal tumors are a rather rare event [24]." "...with the detection of a hitherto unknown mutation in Ha *ras* codon 12, our study extended the overall spectrum of UV-induced *ras* gene mutation, thus emphasizing that unlike most chemical carcinogens with *ras*-activating properties [15], UV light does not seem to preferentially induce the activation of a special *ras* gene or to specifically affect a distinct ras gene codon or-except for the restriction to dipyrimidine bases-a defined base position within a given codon. In other words, UV-mediated mutagenesis of *ras* genes in epidermal keratinocytes appears to be a highly random process at the molecular level."

In addition, Pierceall et al. (1992) studied UV-induced skin tumors in C3H mouse skin and found no A to T transversions at Ha-*ras* codon 61 out of 15 tumors tested.

VII. Evidence that Human Skin Tumors May Contain H-*ras* Mutations

Several papers were found from the early 1990s that report the presence of H-ras mutations in human skin tumors. Much of this work was done in the Ananthaswamy laboratory, and this work is downplayed in more recent (post-2005) publications that unequivocally conclude the p53 mutations are the important events in human skin cancer.

White and Balmain (1988) reported a G to T transversion in codon 12 of the H*ras* gene in a single human BCC. This report was an abstract, and the work was not subsequently published.

Corominas et al. (1989) studied HRAS mutations in 50 human cutaneous SCCs. They found 6 HRAS mutations in 50 SCCs (13%). The most prevalent mutations were AT to TA transversions in codon 61 of HRAS. The authors state that their results "indicate that an activated HRAS oncogene is not sufficient to maintain a neoplastic phenotype and argue against a role of HRAS in the progression of skin tumorigenesis."

Bos (1989) summarized ras oncogenes in human tumors. No ras mutations were reported in human cutaneous BCCs or SCCs.

Van der Schroeff et al. (1990) analyzed the DNA of 30 BCCs and 12 SCCs and found one H-Ras mutation in Codon 61 with a G to T transversion (2%). The authors concluded: "These findings demonstrate that mutational activation of ras genes takes place in skin carcinomas, but the rate at which these mutations occur seems to be relatively low." "Considering the relatively low frequency at which mutated ras genes were found in our study, it seems unlikely that mutational activation of ras genes is a common feature responsible for the induction of skin carcinomas."

Pierceall et al. (1991a) studied 40 human skin cancers and found H-ras mutations that they attributed to ultraviolet light: "We found that 16 of 40 skin tumors analyzed (11 of 24 SCCs and 5 of 16 BCCs) contained identical G-T base changes at the second position of the Ha-ras codon 12." "Since the mutations in the Ha-ras and Ki-ras oncogenes were located opposite potential pyrimidine dimer sites (C-C), it is likely that these mutations were induced by ultraviolet radiation present in sunlight." This work was not repeated in more recent studies from the Ananthaswamy laboratory, and the laboratory has concluded that P53 mutations are major events in the induction of UV-induced skin cancer.

Pierceall et al. (1991b) studied UV irradiation of the c-Ha-*ras*-1 protooncogene *in vitro* and found G to T transversions in H ras codon 12 in foci transformed NIH3T3 cells but not in tumors induced in nude mice. In mice, G to A transitions were found in codon 12. As noted above, this work from the early 1990's was not repeated in this laboratory and the results are downplayed in publications in the 2000s.

Lieu et al. (1991) analyzed 7 human BCCs and 5 human SCCs for mutations at codons 12, 13 and 61 in the Ha-*ras* gene and found one A to T transversion in codon 61 of the Ha-*ras* gene in one BCC and none in the SCCs. They concluded: "In this study we show that the mutations of the Ha-ras gene in spontaneous human epidermal tumors, both benign and malignant, occur at considerably lower frequency than those in the carcinogen-induced tumors of the mice and rabbits."

Ziegler et al. (1993) analyzed 27 human BCCs and found “signature” mutations in p53 in the majority of them. They found only one mutation in H-ras: “Fifty-six percent of tumors contained mutations, and these were UV-like: primarily CC –TT or C - T changes at dipyrimidine sites.” “In contrast to p53, only one mutation was seen in the HRAS, KRAS, and NRAS genes in 10 BCCs, representing a total of 20 kb of sequence on the two alleles (data not shown). That mutation, in codon 12 of NRAS in tumor HB 3, was a C - T substitution at a dipyrimidine site, changing glycine to aspartic acid and capable of activating RAS to an oncogene.” This C to T transition at a dipyrimidine site is a UV “signature” mutation, not a PAH “signature” mutation, which would be a G to T transversion in codon 12 of H-ras.

Nataraji et al. (1995) cited work from their own laboratory indicating that human skin tumors did contain low levels of H-ras mutations. However, they conclude that mutations in p53 are the causative agents, and there is no mention of H-ras mutations in more recent work from the Ananthaswamy laboratory.

“Although mutations in *ras* oncogenes have been implicated in UV carcinogenesis, they do not appear to be as prevalent as mutations in the p53 tumor suppressor gene. In human skin cancers and UV-induced mouse skin cancers, mutations in ras oncogenes occur at 10-40% frequency, [10-12] whereas mutations in the p53 gene occur at 60-100% frequency.[5, 6, 13- 24]”

“Ultraviolet radiation present in sunlight is the primary causative agent in skin cancer. Molecular analysis of human skin cancers originating on sun-exposed body sites and of UV-induced murine skin cancers has identified the p53 tumor suppressor gene as a major target for UV-induced DNA damage, mutation and carcinogenesis. The p53 tumor suppressor gene is by far the most frequently mutated gene in human and UV-induced murine skin cancers. The p53 mutations in UV-induced skin cancers are unique UV "signature" mutations (C to T and CC to TT) that occur predominantly at dipyrimidine sites and are not found in other types of cancer.”

“In human skin cancers and UV-induced mouse skin cancers, mutations in ras oncogenes occur at 10-40% frequency, [10-12] whereas mutations in the p53 gene occur at 60-100% frequency.[5, 6, 13- 24]”

Campbell et al. (1993) stated: “ A recent study from Texas detected codon 12 Harvey-*ras* mutations in 46% of squamous cell carcinomas of the skin and 31 % of basal cell carcinomas.[7] This report, and the relative insensitivity of UNA sequencing as a method for detecting mutations in samples contaminated with normal stromal cells, prompted us to re-examine the role of Ha-*ras* mutations in skin carcinogenesis, using a combination of PCR and restriction fragment length polymorphism of codon 12 of the Ha-ras gene.... We can therefore conclude that Harvey-*ras* codon 12 mutations are rare events in human nonmelanoma skin cancer in the U.K. These results are in keeping with the original studies on *ras* mutations in non-melanoma skin cancers from the Netherlands.[12]”

Wilke et al. (1993) studied the H-ras mutations in 13 human BCCs. An H-ras mutation was found in only one BCC (8%), and it was a G to A transversion in codon 13. This is not a PAH signature mutation.

Spencer et al. (1995) studied H-ras and K-ras mutations in 19 human actinic keratoses and 33 SCCs. They found ras mutations in three of the actinic keratoses and four of the 33 SCCs. They conclude that these ras mutations are likely caused by ultraviolet light: "Ultraviolet light, particularly UV-B (280 to 320 nm), likely plays an important role in the generation of these genetic defects through the formation of pyrimidine dimers and pyrimidine [6-4] pyrimidone lesions.[38] All of the positive tumor samples occurred at sun-exposed sites and the activating mutations were found at codons containing potential pyrimidine dimer sites in the noncoding strand. The power of UV-B to induce these mutations has been shown by in vitro studies in which activating mutations at potential pyrimidine dimer sites were induced in the N-ras gene by directly irradiating isolated DNA.[39] In addition, a wealth of epidemiologic evidence strongly suggests that the UV component of sunlight is the etiologic agent in most human nonmelanoma skin cancers.[30, 31]"

De Gruijl et al. (2001) reported that: "Activating RAS mutations have been reported in a minority of SCC and BCC to various percentages [38,39]. These activating mutations are restricted to the codons 12, 13 and 61, and are not specific of UV radiation. The RAS-pathway may be involved in SCC and BCC, but it is not usually effected through genetic changes in the RAS family of genes."

Melnikova et al. (2005), in a review article from the Anathaswamy laboratory, focus on p53 mutations in human skin cancer. They do refer to the work from their laboratory in the early 1990's that reported H-ras mutations in codon 12, but this work is not repeated in post-2005 papers from this laboratory, and the role of *ras* mutations is minimized. "Further analysis of primary human skin tumors originating on sun-exposed body sites revealed that 16 of 40 skin tumors analyzed (11 of 24 SCCs and 5 of 16 BCCs) contained the identical G→T mutation (glycine to valine) at codon 12 of the H-ras oncogene [125]." "The fact that most ras gene mutations in human skin cancers occur at pyrimidine-rich sequences implies that these sites are probably the targets of UV-induced damage that eventually leads to mutation and transformation."

Benjamin and Anathaswamy (2007), in a more recent review article, refer to H-ras mutations in human skin cancer, but they do not downplay their own 1990 vintage results: "Mutations in ras oncogenes do not appear to be as important as mutations in the p53 tumor suppressor gene in skin cancer development."

Zaravinos et al. (2010) analyzed 12 human SCCs and 15 human BCCs and found one SCC and one BCC carried a G>T transversion at the second position of H*RAS* codon 12. They concluded that HRAS mutations are not common events in nonmelanoma skin cancer in humans. Concerning the reported frequency of occurrence of RAS gene mutations in nonmelanoma skin cancer, the authors stated that the frequency is "often contradictory, depending on the detection methods or geographical criteria employed." They further stated: "Activated HRAS could play a role during

the early stages of SCC or BCC development. However, the low incidence detected suggests that this does not appear to be very significant."

Mauerer et al. (2011) analyzed gene mutations in 31 human cutaneous SCCs and found one HRAS mutation among the 31 tumors. Their results indicated that "HRAS mutations are rare events in the carcinogenesis of cutaneous SCC."

Wang et al. (2011) performed a study of human SCCs and searched for mutations in addition to the well-known p53 mutations: "Squamous cell carcinomas (SCCs) are one of the most frequent forms of human malignancy, but, other than TP53 mutations, few causative somatic aberrations have been identified." There is no discussion of H-ras mutations except to say that they are rare in human skin tumors: "Activating HRAS mutations are highly prevalent in murine SCC-like malignancies but rare in their human counterparts (5, 6)."

Schwarz et al. (2012) stated: "HA-*RAS* mutations are rare in human SCC..."

VIII. Conclusion: Human Skin Cancer Does Not Have a PAH Signature

As noted above, human skin tumors have a unique mutational signature attributed to UV exposure which agrees with the signature of mouse skin tumors induced by UV light. Mouse skin tumors induced by PAH exposures, principally DMBA and BaP in either two-stage initiation/promotion or complete carcinogenesis protocols, contain a distinct and separate mutational signature:

Human Skin Tumors	C to T or CC to TT transitions in p53 at dipyrimidine sites
Mouse UV-Induced Skin Tumors	C to T or CC to TT transitions in p53 at dipyrimidine sites
Mouse BaP–Induced Skin Tumors	A to T transversions in H-*ras* codon 61 or
	G to T transversions in H-*ras* codon 12 or 13

There is scant evidence that PAH-induced mouse skin tumors contain p53 mutations or that human skin tumors contain H-*ras* mutations. As noted above, low levels of p53 mutations in PAH-treated mouse skin have been reported in a hand-full of studies, but the researchers do not implicate them as causal events in mouse skin tumorigenesis. Most of these mutations do not have the PAH mutational signature and/or have no consistent signature at all. Burns et al. (1991) concluded that the p53 mutations were spontaneous in origin. Also, as noted above, low levels of H-ras mutations in human skin tumors have been reported, but these papers are not from the recent literature, and thus are not cited in most recent publications from the responsible laboratories. In addition, the H-ras reported mutations contain the UV mutational signature, not the PAH signature. In conclusion, there is overwhelming evidence in the human population that skin tumors (BCC and SCC) are caused by UV light and do not have a PAH signature.

If EPA's proposed Dermal Slope Factor derived from mouse skin studies were accurate, it would predict that a large fraction of human nonmelanoma skin cancer and more than 100% at some anatomical sites and in some subpopulations was caused by low level dermal exposure to BaP and other PAHs having BaP Relative Potency Factors. While the above studies cannot rule out the possibility that some skin tumors in the past were caused by PAH exposures to the skin or that a *de minimis* fraction of current human skin tumors might have been caused by PAH exposures, there is overwhelming evidence that the vast majority of human skin BCCs and SCC are caused by UV light and not PAHs. There is also almost no evidence in the tumor genotyping literature that PAHs cause any human skin cancer.

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