Influence of *FHIT* on benzo[*a*]pyrene-induced tumors and alopecia in mice: Chemoprevention by budesonide and *N*-acetylcysteine

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The *FHIT* gene has many hallmarks of a tumor-suppressor gene and is involved in a large variety of cancers. We treated A/J mice and (C57BL/6J × 129/SvJ)F_1 (B6/129 F_1) mice, either wild-type or *FHIT*$^{+/-}$, with multiple doses of benzo[*a*]pyrene (B[*a*]P) by gavage. B[*a*]P caused a time-related increase of micronuclei in peripheral blood erythrocytes. Both A/J and B6/129 F_1 mice, irrespective of their *FHIT* status, were sensitive to induction of forestomach tumors, whereas B[*a*]P induced glandular stomach hyperplasia and a high multiplicity of lung tumors in A/J mice only. Preneoplastic lesions of the uterus were more frequent in *FHIT*$^{+/-}$ mice. B6/129 F_1 mice underwent spontaneous alopecia areata and hair bulb cell apoptosis, which were greatly accelerated either by *FHIT* heterozygosity or by B[*a*]P treatment, thus suggesting that *FHIT* plays a role in the pathogenesis of alopecia areata. The oral administration of either budesonide or *N*-acetyl-L-cysteine (NAC) inhibited the occurrence of this inflammatory skin disease. In addition, these agents prevented B[*a*]P-induced glandular stomach hyperplasia and decreased the size of both forestomach tumors and lung tumors in A/J mice. Budesonide also attenuated lung tumor multiplicity. In B6/129 F_1 mice, NAC significantly decreased the proliferating cell nuclear antigen in lung tumors. Both budesonide and NAC inhibited B[*a*]P-induced forestomach tumors and preneoplastic lesions of the respiratory tract in B6/129 F_1 mice. In conclusion, heterozygosity for *FHIT* affects susceptibility of mice to spontaneous alopecia areata and B[*a*]P-induced preneoplastic lesions of the uterus and does not alter responsiveness to budesonide and NAC.

alopecia areata | lung tumors | stomach tumors

The *FHIT* gene is thus far the only example of a gene at a constitutive fragile region, and it shows many hallmarks of a tumor-suppressor gene (1). *FHIT* is altered by deletion or translocation in a large fraction of cancers, among which are lung and gastric cancer (2–4). The murine *FHIT* gene is similar in sequence, location, and fragility to its human homologue (5), which suggests that *FHIT* mutant mice might provide a model to study the role of the *FHIT* pathway in the development of cancer and possibly of other diseases. *FHIT*-deficient mice, either *FHIT*$^{+/-}$ or *FHIT*$^{-/-}$, were established by inactivating one *FHIT* allele in mouse embryonic stem cells. These mice displayed an elevated frequency of "spontaneous" tumors and chemically induced tumors (6, 7). The *FHIT*-deficient mouse model has also been used to prevent tumor development by gene transfer (8, 9).

One objective of the present study was to compare the susceptibility of (C57BL/6J × 129/SvJ)F_1 (B6/129 F_1) mice, either wild type (*FHIT*$^{+/+}$) or heterozygous for *FHIT* (*FHIT*$^{+/-}$), to benzo[*a*]pyrene (B[*a*]P), a prototype of genotoxic and carcinogenic polycyclic aromatic hydrocarbon (PAH). B[*a*]P diol epoxide has recently been shown to down-regulate *FHIT* expression, presumably as the result of different signaling pathways triggered by specific DNA lesions (10). In parallel, we used A/J mice as a control, because this mouse strain has been extensively used in the lung tumor assay (11), a medium-term bioassay for lung tumorigenesis. A/J mice carry the pulmonary adenoma susceptibility 1 (Pas1) locus, a major locus affecting predisposition to lung cancer in mice (12) and producing the EcoRI-generated 0.55-kb K-ras fragment associated with high susceptibility to lung tumor development (13).

Another major goal of this study was to assess the protective effects of the two chemopreventive agents budesonide and *N*-acetyl-L-cysteine (NAC). Budesonide is a glucocorticoid, a family of compounds that are effective cancer chemopreventive agents in animal models but can have side effects in humans (14). In particular, the studies performed by Wattenberg and colleagues (15–17) showed that budesonide, given either in the diet or by aerosol, inhibits the formation of B[*a*]P-induced lung tumors in A/J mice at all stages of tumor development, from hyperplasia to cancer. The expression of a number of genes was modulated by budesonide in the lung tumors developed in B[*a*]P-treated A/J mice (18). The protective effect of oral budesonide against lung tumors induced by intraperitoneal B[*a*]P was also observed in A/J mice, either wild type or those carrying germ-line mutations in *P53* and/or *Ink4A/Arf* genes (19). Moreover, budesonide delayed the appearance of lung tumors in strain A mice treated with vinyl carbamate and decreased their growth and progression to carcinomas (20).

NAC works as an analogue and a precursor of intracellular L-cysteine and reduced glutathione (GSH). The safety of this drug in humans is supported by >40 years of clinical use, mainly as a mucolytic agent but also as an antidote against acute intoxication (21, 22). A number of experimental studies in animal models performed during the last 20 years and phase II clinical trials, along with mechanistic considerations, provide evidence for the potential ability of this thiol compound to inhibit oxidative, genotoxic, and carcinogenic effects (reviewed in refs. 21 and 22). As an indicator of safety at the molecular level, oral NAC did not substantially change the baseline expression of multiple genes but attenuated the alterations induced by cigarette smoke in both mice (23) and rats (24). Among other protective effects, dietary NAC attenuated B[*a*]P-induced pathology of the liver and forestomach hyperplasia and papillomas in *P53* heterozygous TG.AC (v-Ha-ras) bitransgenic mice (25).

An incidental discovery during the progress of the reported study herein was that B6/129 F_1 mice spontaneously developed areas of alopecia, whose formation was greatly accelerated in

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Abbreviations: B[a]P, benzo[a]pyrene; Fhit, fragile histidine triad; MN, micronucleated; NAC, *N*-acetyl-L-cysteine; NCE, normochromatic erythrocytes; PCNA, proliferating cell nuclear antigen.

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Table 1. Yield of forestomach tumors and glandular stomach hyperplasia in mice related to strain, *Fhit* status, and treatment

Mouse strain	*Fhit* status	Treatment	Forestomach tumors, total		Forestomach tumors, >1 mm		Glandular stomach hyperplasia incidence, %
			Incidence, %	Multiplicity, mean ± SE	Incidence, %	Multiplicity, mean ± SE	
A/J	+/+	Controls	0/20 (0)	0	0/20 (0)	0	2/20 (10.0)
		B[*a*]P	18/20 (90.0)*	2.5 ± 0.46*	18/20 (90.0)*	2.4 ± 0.47*	7/20 (35.0)†
		B[*a*]P + Bud	18/19 (94.7)*	3.3 ± 0.50*	18/19 (94.7)*	2.8 ± 0.39*	0/19 (0)‡
		B[*a*]P + NAC	17/20 (85.0)*	2.9 ± 0.42*	17/20 (85.0)*	2.7 ± 0.40*	0/17 (0)‡
B6/129 F_1	+/+	Controls	1/14 (7.1)	0.1 ± 0.08	0/14 (0)	0	1/14 (7.1)
		B[*a*]P	13/17 (76.5)*	3.9 ± 0.71*	13/17 (76.5)*	2.0 ± 0.44*	3/17 (17.6)
		B[*a*]P + Bud	13/19 (68.4)*	1.9 ± 0.46*§	12/19 (63.2)*	1.3 ± 0.40*	0/19 (0)
		B[*a*]P + NAC	14/20 (70.0)*	1.6 ± 0.15*¶	11/20 (55.0)*	1.3 ± 0.34*	0/20 (0)
B6/129 F_1	+/−	Controls	1/20 (5.0)	0.1 ± 0.05	0/20 (0)	0	2/20 (10.0)
		B[*a*]P	22/22 (100)*	3.3 ± 0.41*	20/22 (90.9)*	2.0 ± 0.19*	2/22 (9.1)
		B[*a*]P + Bud	11/20 (55.0)*¶	0.9 ± 0.24*¶	9/20 (45.0)*¶	0.7 ± 0.20¶	0/20 (0)
		B[*a*]P + NAC	13/22 (59.1)*¶	1.7 ± 0.42*§	9/22 (40.9)*¶	0.8 ± 0.25¶	0/22 (0)

Statistically significant differences: *, $P < 0.001$ and †, $P < 0.05$ compared with controls; ‡, $P < 0.01$; §, $P < 0.05$; and ¶, $P < 0.001$, compared with mice treated with B[a]P only. Bud, budesonide.

FHIT$^{+/-}$ mice. NAC and, even more strikingly, budesonide inhibited both the spontaneous alopecia in *FHIT*$^{+/-}$ mice and the B[*a*]P-induced alopecia in wild-type mice. Moreover, in A/J and/or B6/129 F_1 mice, these chemopreventive agents significantly attenuated the induction of preneoplastic lesions and tumors by B[*a*]P.

Results

Body Weights and Survival. No treatment significantly affected the body weight gain either in A/J or B6/129 F_1 mice (data not shown). Irrespective of the mouse strain, *FHIT* status, and treatment, almost all mice (95.1%) were still alive at the end of the experiment. The number of mice surviving within each experimental group can be inferred from the incidence data reported in Tables 1 and 2.

Cytogenetic Monitoring. The results relative to the periodic cytogenetic monitoring in the peripheral blood of the variously treated mice are shown in Fig. 1. In control mice, which were treated with corn oil, the levels of micronucleated (MN) normochromatic erythrocytes (NCE) were >2-fold higher in A/J mice than in B6/129 F_1 mice. Both A/J and B6/129 F_1 mice responded to the multiple administrations of B[*a*]P by increasing the frequency of MN NCE. This effect started to be statistically significant after 10–12 days of B[*a*]P administration and reached a plateau after ≈3 weeks. The maximum increase in B[*a*]P-treated mice, compared with controls, was ×2.2 in A/J mice, ×3.0 in wild-type B6/129 F_1 mice, and ×3.2 in *FHIT*$^{+/-}$ B6/129 F_1 mice and thereafter tended to decline by the end of the experiment. Irrespective of the mouse strain and *FHIT* status, neither budesonide nor NAC affected the frequency of B[*a*]P-induced MN NCE.

Spontaneous and B[*a*]P-Induced Alopecia Areata. A number of B6/129 F_1 mice developed evident alopecia areas. In Fig. 2, examples are shown in which the lesions are distinguished according to their localization on the muzzle (A), head (B), neck (C), and back (D). Some mice had single localizations, whereas other mice had multiple localizations of variable intensity. Fig. 2 also shows the time course induction of alopecia related to *FHIT* status and treatments. The mice were checked for alopecia 0, 29, 43, 57, 72, 101, 116, 134, 148, 176, 198, 205, and 276 days

Table 2. Yield and characteristics of lung tumors in mice related to strain, *Fhit* status, and treatment

Mouse strain	Parameter	Treatment of mice			
		Controls	B[*a*]P	B[*a*]P + budesonide	B[*a*]P + NAC
A/J	Incidence, %	3/20 (15.0)	20/20 (100)*	19/19 (100)*	20/20 (100)*
	Multiplicity, mean ± SE	0.2 ± 0.12	10.4 ± 1.23*	5.9 ± 0.71*†	9.4 ± 1.06*
	Diameter, mm, mean ± SE	1.5 ± 0.29	1.2 ± 0.03	0.9 ± 0.04†	1.0 ± 0.03†
B6/129 F_1 wild type	Incidence, %	0/14 (0)	3/17 (17.6)	4/20 (20.0)	2/19 (10.5)
	Multiplicity, mean ± SE	0	0.3 ± 0.19	0.5 ± 0.21	0.3 ± 0.27
	Diameter, mm, mean ± SE	NTA	1.8 ± 0.58	1.2 ± 0.17	1.2 ± 0.17
	P53 in tumors, %	NTA	11.3 ± 3.55	8.0 ± 1.00	8.5 ± 2.19
	PCNA in tumors, %	NTA	29.2 ± 3.25	23.3 ± 1.45	20.8 ± 2.02‡
	Apoptotic cells in tumors, %	NTA	5.5 ± 1.04	6.4 ± 0.57	5.9 ± 0.65
B6/129 F_1 *Fhit*$^{+/-}$	Incidence, %	0/20 (0)	6/23 (26.1)§	5/23 (21.7)§	5/20 (25.0)§
	Multiplicity, mean ± SE	0	0.4 ± 0.15	0.4 ± 0.16	0.3 ± 0.13
	Diameter, mm, mean ± SE	0	1.5 ± 0.39	1.3 ± 0.23	1.1 ± 0.24
	P53 in tumors, %	NTA	11.0 ± 1.71	11.1 ± 2.22	9.0 ± 1.39
	PCNA in tumors, %	NTA	27.7 ± 1.79	24.2 ± 1.69	21.0 ± 2.46‡
	Apoptotic cells in tumors, %	NTA	4.9 ± 0.62	5.2 ± 0.91	4.4 ± 0.71

Statistically significant differences: *, $P < 0.001$ and §, $P < 0.05$ compared with controls; †, $P < 0.01$ and ‡, $P < 0.05$ compared with mice treated with B[a]P only. NTA, no tumor available.

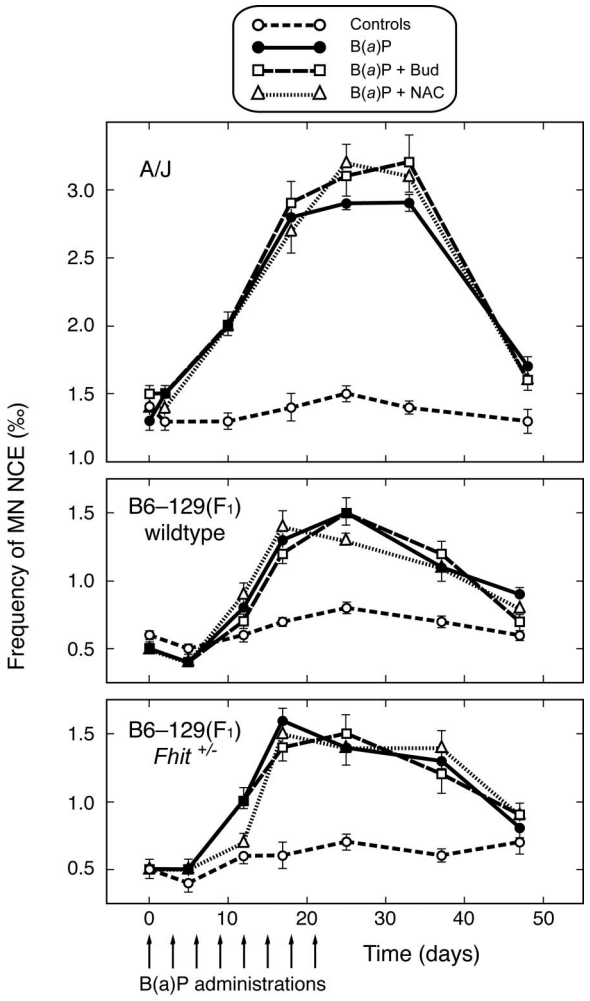

Fig. 1. Frequency of MN NCE in peripheral blood samples collected after various time intervals in A/J, wild-type, and *FHIT*$^{+/-}$ B6/129 F_1 mice, which were untreated, received multiple administrations of B[*a*]P, or were cotreated with budesonide (Bud) or NAC.

after the first B[*a*]P administration. Within wild-type mice, the control animals started loosing their agouti hair after 101 days, and all control mice were affected by alopecia areas after 176 days. B[*a*]P strongly accelerated this process until it affected 100% of mice after only 57 days, with statistically significant differences compared with controls. Coadministration of budesonide significantly decreased the frequency of alopecia, not only compared with B[*a*]P-treated mice from 57 days onward, but even compared with untreated controls from 134 days onward. Although less strikingly than budesonide, NAC also attenuated B[*a*]P-induced alopecia areata after 57–72 days. After 200 days, all mice, irrespective of *FHIT* status and treatment, suffered from alopecia areata. Almost all *FHIT*$^{+/-}$ mice were still affected after 276 days.

As is evident in Fig. 2, untreated *FHIT*$^{+/-}$ mice were much more prone than untreated wild-type mice to developing "spontaneous" alopecia throughout almost the whole duration of the experiment, with significant differences related to *FHIT* status after 29–148 days. In mutant mice, B[*a*]P did not further affect alopecia formation compared with controls. The chemopreventive agents tested exerted strong protective effects on induction of alopecia compared with either controls or B[*a*]P-treated mice. In fact, both budesonide and NAC significantly inhibited alopecia after 29–198 days.

As assessed by immunohistochemical analysis of apparently "healthy" skin fragments not affected by alopecia that were collected from five mice per experimental group, virtually all cells in the basal and granular layers of epidermis, as well as in the hair bulbs and sebaceous glands, were positive for fragile histidine triad (Fhit). Only 0.1–0.4% of cells were positive for either mutated or inactivated P53, without appreciable differences related to *FHIT* status of mice and treatment with B[*a*]P and chemopreventive agents. Hair bulb cells were analyzed for the detection of apoptosis by the TUNEL method. In wild-type B6/129 F_1 mice, the proportion of apoptotic cells was significantly enhanced after treatment with B[*a*]P (15.3%) compared with control mice (1.2%). The effect of B[*a*]P was significantly attenuated by cotreatment either with budesonide (4.3%) or NAC (9.4%). By comparison with wild-type mice, the background apoptotic frequency was much higher in untreated *FHIT*$^{+/-}$ mice (13.5%) and was not significantly increased by B[*a*]P administration (16.8%). Both budesonide (3.8%) and NAC (5.7%) remarkably decreased apoptosis in hair bulb cells, with significant differences compared not only with B[*a*]P-treated mice but even with untreated mice.

Forestomach Tumors and Glandular Stomach Hyperplasia. Table 1 shows the incidence and multiplicity of macroscopically visible forestomach tumors and the incidence of microscopically detectable hyperplasia of the glandular stomach epithelium in the variously treated mice.

B[*a*]P induced a high incidence and multiplicity of total forestomach tumors in both A/J and B6/129 F_1 mice, irrespective of *FHIT* status. In A/J mice, budesonide and NAC did not affect induction of tumors by B[*a*]P, but both agents were successful in decreasing the size of forestomach tumors to a significant extent. In fact, the diameter of these tumors (mean ± SE) in A/J mice was 2.9 ± 0.28 mm in B[*a*]P-treated mice, 1.9 ± 0.15 in mice cotreated with budesonide ($P < 0.01$, compared with mice treated with B[*a*]P only), and 2.2 ± 0.17 in mice cotreated with NAC ($P < 0.01$). However, in B[*a*]P-treated B6/129 F_1 mice, both budesonide and NAC significantly decreased tumor multiplicity, which was more than halved in both wild-type and *FHIT*$^{+/-}$ mice receiving either chemopreventive agent. Moreover, both budesonide and NAC significantly decreased the tumor incidence in B[*a*]P-treated *FHIT*$^{+/-}$ mice.

The protective effect of the chemopreventive agents tested was also clearly evident when comparing only forestomach tumors whose diameter was >1 mm, which are more likely to be true tumors rather than preneoplastic lesions. In fact, as shown in Table 1, the B[*a*]P-induced tumor multiplicity was reduced 1.5-fold by both budesonide and NAC in wild-type mice (nonsignificant), whereas it was significantly reduced by budesonide (2.9-fold) and NAC (2.5-fold) in *FHIT*$^{+/-}$ mice. In any case, multiplicity of B[*a*]P-induced forestomach tumors was not significantly different in wild-type mice and in *FHIT*$^{+/-}$ mice cotreated either with budesonide or NAC.

The histopathological analysis revealed that, in addition to forestomach tumors, B[*a*]P induced a significant increase of glandular stomach hyperplasia (3.5-fold) in A/J mice. Both budesonide and NAC completely prevented this kind of lesion (Table 1). Moreover, irrespective of the mouse strain and *FHIT* status, B[*a*]P increased the incidence of microscopically detectable keratoses and hyperplasias of the forestomach (data not shown).

Lung Lesions. As shown in Table 2, despite the termination of mice earlier in the experiment, A/J mice were much more susceptible to the induction of lung tumors than B6/129 F_1 mice. Compared with controls, the increase of lung tumor incidence in B[*a*]P-treated B6/129 F_1 mice was statistically significant in *FHIT*$^{+/-}$ mice only. However, the difference between wild-type and *FHIT*$^{+/-}$ mice was modest and not statistically significant. As

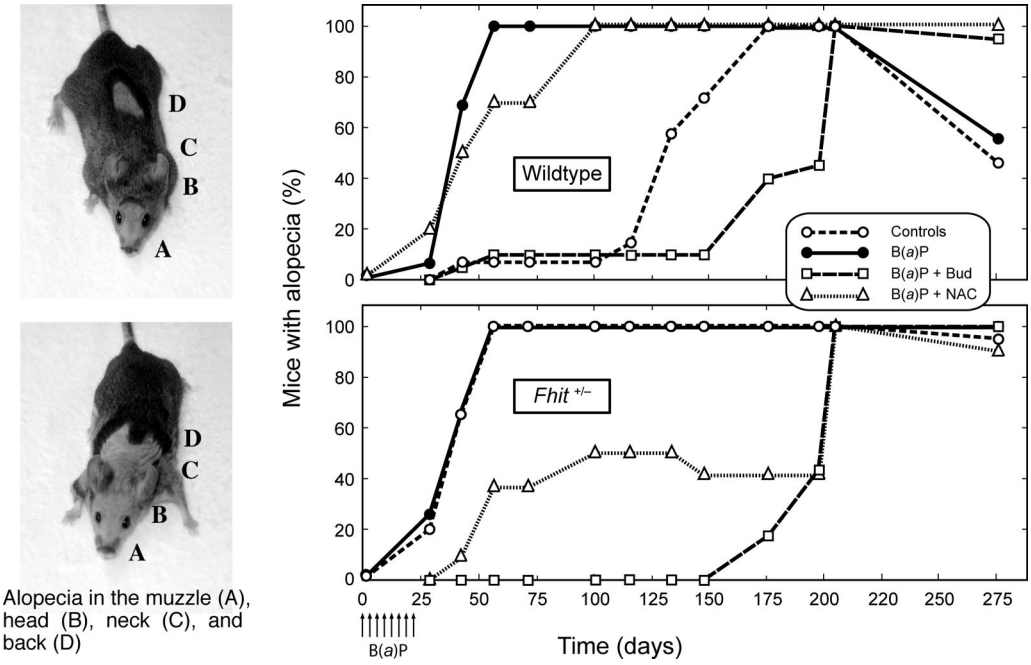

Fig. 2. Appearance of alopecia areata in B6/129 F_1 mice and time course formation of alopecia related to *FHIT* status and treatment of mice.

assessed in B6/129 F_1 mice, 64.3% of B[*a*]P-induced lung tumors were solid adenomas, 23.8% were mixed adenomas, and 11.9% were papillary adenomas. The histopathological type was affected neither by *FHIT* status nor by administration of either chemopreventive agent.

In A/J mice, budesonide significantly decreased lung tumor multiplicity, and both budesonide and NAC significantly decreased the lung tumor size. The low incidence and multiplicity of lung tumors in B[*a*]P-treated mice were not significantly changed in B6/129 F_1 mice cotreated with the chemopreventive agents, regardless of *FHIT* status. Both budesonide and NAC decreased the mean diameter of tumors, but, due to the larger variability in size compared with tumors in A/J mice, these differences were not statistically significant.

Approximately 10% of cells composing lung tumors in B[*a*]P-treated B6/129 F_1 mice harbored the P53 protein in the nucleus, 21–29% had detectable proliferating cell nuclear antigen (PCNA), and 4–6% were apoptotic. Interestingly, PCNA was significantly decreased in the tumors from both wild-type and $FHIT^{+/-}$ mice cotreated with NAC (Table 2). The immunohistochemical analysis of 25 lung tumors induced by B[*a*]P in B6/129 F_1 mice showed mixed areas of positivity and negativity for Fhit protein in each tumor, without appreciable differences related to either *FHIT* status or treatment (data not shown).

In addition to lung tumors, the histopathological analyses performed in B6/129 F_1 mice revealed the presence of other lesions in the respiratory tract. In particular, there were two cases of severe emphysema in both wild-type (10.5%) and $FHIT^{+/-}$ (8.7%) mice treated with B[*a*]P only, and one case was detected in $FHIT^{+/-}$ controls (5.0%). These differences were not statistically significant. Whereas no hyperplasia of the bronchial epithelium was observed in control mice, this alteration occurred in B[*a*]P-treated mice, both wild type (29.4%; $P = 0.01$) and $FHIT^{+/-}$ (30.4%; $P < 0.01$). The B[*a*]P-induced hyperplasia of the bronchial epithelium was significantly inhibited either by NAC in both wild-type (no case; $P = 0.01$, compared with mice treated only with B[*a*]P) and $FHIT^{+/-}$ (one case; $P < 0.05$) mice or budesonide (no case in either wild-type or $FHIT^{+/-}$ mice; $P < 0.01$). In addition, whereas no hyperplasia of alveolar walls was detected in control mice, B[*a*]P induced this type of lesion in both wild-type (52.8%; $P < 0.001$) and $FHIT^{+/-}$ (73.9%; $P < 0.001$) mice. A protective effect toward this B[*a*]P-induced damage was only observed in $FHIT^{+/-}$ mice cotreated with budesonide (21.7%; $P < 0.01$).

Histopathological Alterations in Other Organs. After complete necropsy and histopathological analysis of macroscopically suspect lesions of B6/129 F_1 mice, only a single case of cystic hyperplasia of the uterus was detected in B[*a*]P-treated wild-type mice. In contrast, preneoplastic lesions of the uterus (cystic hyperplasia and leiomyomas) were detected in 21.2% of $FHIT^{+/-}$ mice ($P < 0.001$), irrespective of treatment with the chemopreventive agents. In addition, liver hemangiomas were detected in 4.5% of these mice.

Discussion

The present study evaluated the susceptibility of A/J mice and B6/129 F_1 mice, either wild-type or $FHIT^{+/-}$, to a variety of pathological conditions, either "spontaneous" or induced by B[*a*]P. Moreover, the chemopreventive effects of budesonide and NAC were investigated.

During the progress of this study, B6/129 F_1 mice underwent spontaneous alopecia areata, which was greatly accelerated either by *FHIT* heterozygosity or treatment with B[*a*]P. In parallel, the proportion of apoptotic cells in the hair bulbs was greatly enhanced either by heterozygosity for *FHIT* or administration of B[*a*]P. Alopecia areata is a nonscarring, inflammatory form of patchy hair loss that is determined by a combination of genetic factors and environmental stimuli (26). Among the multiple genes involved in its pathogenesis, *P53* has been shown to be involved in the control of apoptosis in the hair follicle during physiological regression (27). The results of our study, highlighting an enhanced hair bulb cell apoptosis and an increased alopecia in untreated *FHIT*-deficient mice, suggest that *FHIT* plays a protective role in alopecia areata. Interestingly, *FHIT*-deficient B6/129 F_1 mice can also develop tumors of sebaceous glands, similar to those observed in Muir–Torre syndrome in humans (7). B6/129 F_1 agouti mice are derived from black C57BL/6J mice, and C57BL/6 and related strains are

susceptible to hair loss. For instance, alopecia-dependent apoptosis was induced in C57BL/6 mice either treated with doxorubicin (28), an anticancer drug that typically induces oxidative DNA damage, or exposed whole-body to environmental cigarette smoke (29). The involvement of environmental factors in the induction of alopecia areata is further supported by the present finding that B[*a*]P, a typical component of cigarette smoke and other complex mixtures resulting from combustion processes, induced hair loss in wild-type mice. Also in humans, hair follicle cells have been shown to be able to convert B[*a*]P to ultimate DNA-damaging and carcinogenic metabolites (30).

Irrespective of *FHIT* status, no lung tumors or glandular stomach hyperplasia were observed in untreated B6/129 F_1 mice, and the spontaneous incidence of forestomach tumors was very low. These findings are in line with the conclusion that the incidence of spontaneous lung tumors is approximately the same in wild-type and *FHIT*-deficient B6/129 F_1 mice (6). In addition, the baseline frequency of MN NCE, monitored at periodic intervals in peripheral blood, was particularly low in this mouse strain.

Multiple administrations of B[*a*]P by gavage induced, in both A/J and B6/129 F_1 mice, an evident increase of MN NCE in peripheral blood, which reflects the induction of cytogenetic damage in bone marrow cells. Consistent with the fact that treatment with B[*a*]P lasted 28 days and that the half-life of NCE in mice is ≈30 days (31), the levels of B[*a*]P-induced MN NCE declined by the end of the monitoring period. As expected, B[*a*]P induced a high multiplicity of lung tumors in all treated A/J mice, whereas both incidence and multiplicity of lung tumors were modest in B6/129 F_1 mice. Note that these mice are derived from C57BL/6 mice, which are rather insensitive to the induction of lung tumors (32). In addition, B[*a*]P caused hyperplasia of the glandular stomach in an appreciable proportion of A/J mice, whereas these histopathological alterations were infrequent in B6/129 F_1 mice. Both mouse strains were conversely sensitive to the induction of forestomach keratoses, hyperplasias, and tumors by B[*a*]P. In addition, irrespective of *FHIT* status, the oral administration of B[*a*]P to B6/129 F_1 mice caused hyperplasia of both bronchial epithelium and alveolar walls. Interestingly, a B[*a*]P-related formation of preneoplastic lesions of the uterus was only evident in $FHIT^{+/-}$ mice.

Thus, on the whole, the yield of both forestomach tumors and lung tumors by B[*a*]P does not appear to be affected by heterozygosity for *FHIT*. However, $FHIT^{+/-}$ mice were more sensitive than wild-type mice to the induction by B[*a*]P of uterus and liver pathological lesions, which suggests a possible protective role of *FHIT* in these organs. It should be noted that *FHIT* inactivation is an early event in carcinogenesis of the endometrium (33) and that occurrence of liver hemangiomas was described in untreated *FHIT*-deficient mice (6). In previous studies, $FHIT^{+/-}$ mice had been shown to be more susceptible than their wild-type counterparts to the induction of forestomach tumors by *N*-nitrosomethylbenzylamine (NMBA) (6), whereas there was no difference in the induction of lung tumors by 4-methylnitrosamino-1,3-pyridyl-1-butanone (NNK) in *FHIT* heterozygous mice (34).

The oral administration of the chemopreventive agents NAC and budesonide inhibited hair bulb cell apoptosis and formation of alopecia areata. Moreover, budesonide and NAC exerted a variety of protective effects on preneoplastic and neoplastic alterations induced by B[*a*]P. The protective effect of budesonide is consistent with the well known antiinflammatory properties of glucocorticoids (14). Among the variety of mechanisms of NAC, this thiol compound has antiinflammatory properties and inhibits triggering of apoptosis consequent to DNA damage and redox imbalances (22). NAC has been shown to prevent alopecia induced by either doxorubicin (28) or cigarette smoke (29) in C57BL/6 mice or by 2-chloroethyl ethyl sulfide in guinea pigs (35). Interestingly, NAC was able to inhibit the loss of Fhit protein induced by cigarette smoke in the bronchial epithelium of Sprague–Dawley rats, whereas other chemopreventive agents, including oltipraz, 5,6-benzoflavone, phenethyl isothiocyanate, and indole-3-carbinol, were ineffective (36). As an antioxidant, nucleophile, and scavenger of free radicals, NAC is likely to suppress the stimuli that alter the *FHIT* gene and cause the loss of Fhit protein. In addition, because Cu(II) is a strong inhibitor of the enzymatic activity of Fhit due to its reaction with Cys-39, which bears the only thiol group in the Fhit monomer (37), protection of Fhit by NAC may also be ascribed to the ability of thiols to react with Cu(II) and other metals (38).

In conclusion, heterozygosity for *FHIT* does not appear to confer an increased susceptibility of B6/129 F_1 mice to B[*a*]P tumorigenicity in lung and stomach but renders the animals more susceptible to development of spontaneous alopecia areata and B[*a*]P-induced preneoplastic lesions of the uterus. Budesonide and NAC are able to inhibit B[*a*]P-induced forestomach tumors and preneoplastic lesions in the respiratory tract. In addition, they prevent both spontaneous and B[*a*]P-induced alopecia areata, an inflammatory skin disease in which *FHIT* appears to play an evident protective role.

Materials and Methods

Mice. Eighty female A/J mice weighing 18–19 g at the start of the experiment were commercially available (Harlan Italy, S. Pietro al Natisone, Italy). A total of 163 female B6/129 F_1, 74 wild-type, and 89 $FHIT^{+/-}$ mice were bred at the Kimmel Cancer Center (Thomas Jefferson University, Philadelphia) and shipped to the University of Genoa. They weighed 24–27 g at the start of the experiment. The mice were housed in Makrolon cages on sawdust bedding and were maintained on standard mouse chow (MIL Morini, San Polo d'Enza, Italy) and tap water ad libitum. The temperature of the animal room was 23 ± 2°C, with a relative humidity of 55%, ventilation accounting for 15 air renewal cycles per hour, and a 12-h light–dark cycle. The housing and treatment of animals were in accordance with our national and institutional guidelines.

Chemicals. B[*a*]P and budesonide were purchased from Sigma, and NAC was purchased from Zambon (Bresso, Italy) in the form of a commercially available product (Fluimucil).

Treatment of Mice. After a 2-week acclimatization, A/J, B6/129 F_1 wild-type, and B6/129 F_1 $FHIT^{+/-}$ mice were divided into four groups, each composed of 14–23 animals. The mice belonging to the first group (controls) were treated with corn oil given by gavage (0.1 ml). The mice belonging to the second group were treated with B[*a*]P dissolved in corn oil (0.1 ml) and given by gavage in eight doses (1 mg per dose) twice per week for 4 weeks. The mice belonging to the third group (B[*a*]P plus budesonide) were treated with B[*a*]P in the same way as mice in the second group and received budesonide with the diet (2.4 mg/kg diet), which was prepared once per week. The mice belonging to the fourth group (B[*a*]P plus NAC) were treated with B[*a*]P in the same way as mice in the second group and received NAC with the drinking water at a calculated daily intake of 1 g/kg body weight. Treatment with the two chemopreventive agents started 3 days before the first B[*a*]P administration and continued until the end of the experiment. The mice were inspected daily for their general appearance and weighed individually at monthly intervals. All A/J mice were killed 7 months after the first B[*a*]P administration, whereas all B6/129 F_1 mice were killed 11 months after the first B[*a*]P administration to allow for a better growth of lung tumors. The mice were killed by cervical dislocation after deep anesthesia with diethyl ether. Stomach, lungs, kidneys, and liver were collected from all mice. A complete necropsy was performed, and tissues with macroscopically visible alterations were subjected to standard histopathological analysis.

Cytogenetic Monitoring. At periodic intervals (see Fig. 1), samples of blood were collected from the tail lateral vein of 10 mice per group. Duplicates of smears of peripheral blood were stained with May–Grünwald–Giemsa. An average of 50,000 NCE were scored per mouse, accounting for a total of 42 million NCE scored for the presence of MN. The results are expressed as MN frequency per 1,000 NCE.

Forestomach Tumors and Glandular Stomach Hyperplasia. The forestomach of each mouse was cut longitudinally, and forestomach tumors were detected with the aid of a stereomicroscope. The forestomach and glandular stomach of each mouse were cut into 10 sections each and subjected to standard microscopic analysis to evaluate possible histopathological alterations.

Lung Tumors. The lungs were immersed in buffered formalin, and, after 48 h, the tumors were detected on the lung surface with the aid of a stereomicroscope. The two main diameters of each tumor were measured with a linear micrometer, and the mean diameter was calculated. All tumors were subjected to standard histopathological analysis.

P53, Fhit, PCNA, and Apoptosis. Mutated or inactivated P53 protein was detected in skin fragments and lung tumors by immunohistochemistry using the CM-5 polyclonal antibody (NovoCastra, Newcastle upon Tyne, U.K.). Positivity for Fhit in skin cells and lung tumors was evaluated by immunohistochemistry using a rabbit anti-Fhit polyclonal antibody, kindly supplied by Kay Huebner (Ohio State University Comprehensive Cancer Center, Columbus), at a final dilution of 1:2,000. Formalin-fixed, paraffin-embedded sections were routinely processed by using the HistoMouse-SP kit (Zymed Laboratories), according to the manufacturer's instructions. The slides were scored at a magnification of ×400, and 1,000 cells per slide were examined.

PCNA was detected by immunohistochemistry using the PCNA staining kit (Zymed Laboratories). This kit is based on an anti-PCNA monoclonal antibody (clone PC10) and uses avidin/biotinylated peroxidase complex (ABC) technology. The slides were scored at a magnification of ×400, and 1,000 cells per slide were examined.

The frequency of apoptotic cells was evaluated by the TUNEL method with two different commercially available kits. For skin cells, we used the Dermatacs *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD), and, for lung tumor cells, we used the Tacs XL Blue Label *in situ* apoptosis detection kit (Trevigen). Both kits were used according to the manufacturer's instructions. The slides were scored at a magnification of ×400, and 1,000 cells per mouse were examined.

Statistical Analyses. Comparisons of treatment-related differences regarding frequency of alopecia, incidence of tumors and preneoplastic lesions, and frequency of P53-positive, PCNA-positive, and apoptotic cells were made by χ^2 analysis. Comparisons regarding quantitative data, expressed as means ± SE, including body weight, tumor multiplicity, and size of tumors, were made by Student's *t* test for unpaired data. Differences with $P < 0.05$ were taken as statistically significant.

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