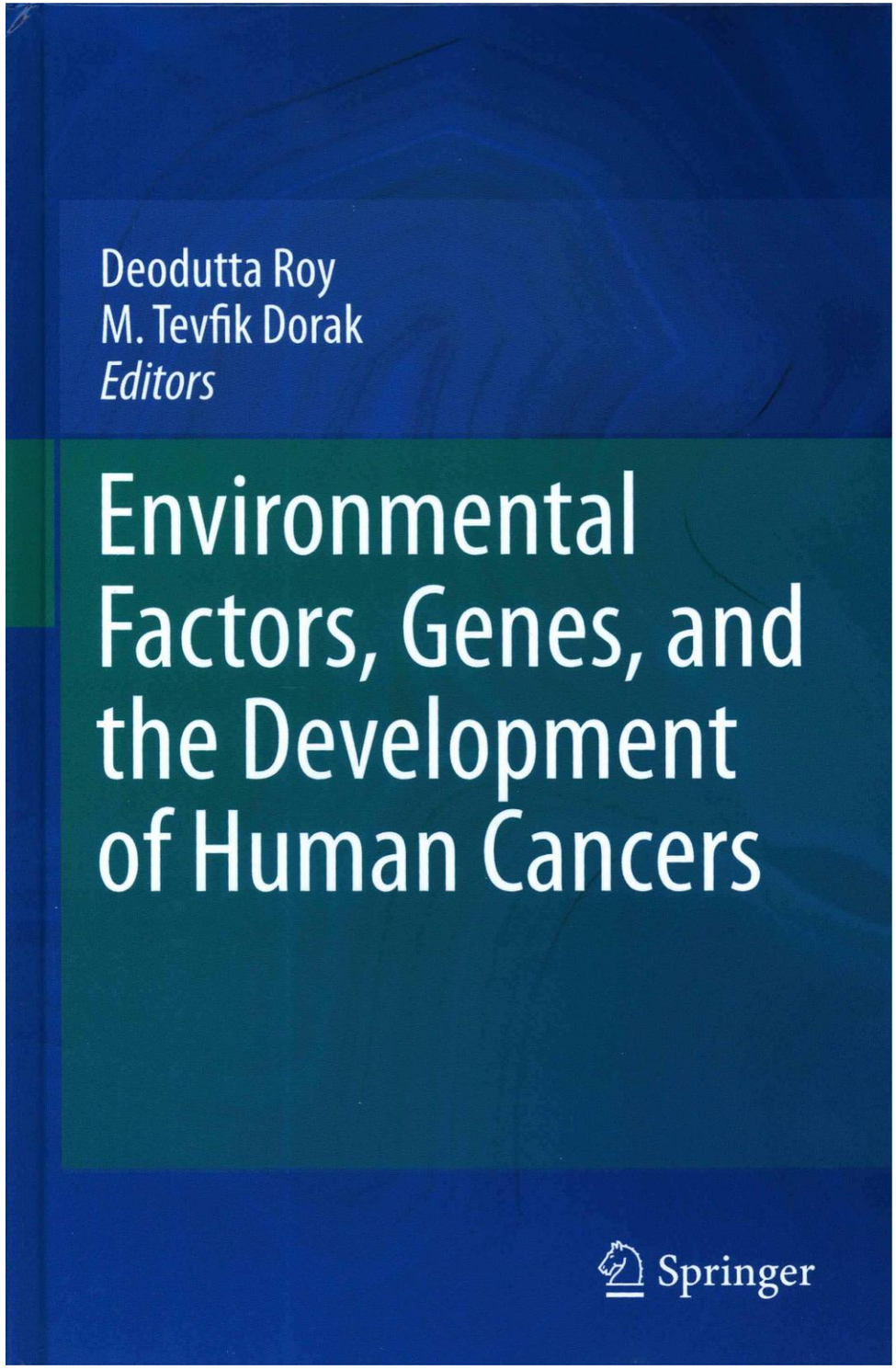

Chapter 15
Betel Nut and Susceptibility to Cancer

Rajesh N. Sharan and Yashmin Choudhury

Abstract Betel nut is a widely masticated natural product, which is consumed by over 600 million people across the globe. The ancient habit of betel nut chewing, either as dry or raw/wet nut, in association with betel leaf and a host of region specific additives, including chewing tobacco, is believed to be an important etiological factor for human cancer. Alkaloids and their betel nut specific nitrosamine derivatives produced upon metabolic activation interact with DNA and other cellular targets to produce highly variable mutagenic, genotoxic, cytostatic, immunostatic and teratogenic effects. At molecular level the betel nut or its constituents strongly influence gene expression patterns, especially that of tumor suppressor genes. Structural damage to nucleus and mitochondria, etc. are also induced. The review dwells upon these aspects of betel nut induced carcinogenesis to show that genetic susceptibility to cancer through generations progressively increased due to exposure to betel nut.

Keywords Betel nut specific nitrosamine derivatives and alkaloids · Mutagenic and genotoxic effects · Tumor suppressor genes · Susceptibility to oral cancer

15.1 Introduction

Areca nut is the seed of fruit of a tropical palm, *Areca catechu* L (Fig. 15.1a). It forms the most basic ingredient of a variety of widely used social and habitual masticatory products, which are often wrapped in the leaf of another tropical creeper, *Piper betle* L., commonly known as the betel leaf. Hence, the *Areca* nut is more commonly known as betel nut (BN) (Warnakulasuriya, 2002). The earliest use of BN as a masticatory by humans has been mentioned by Theophrastus in scripts dating around 430 BCE (Before Common Era), which described use of *Areca* nut as a

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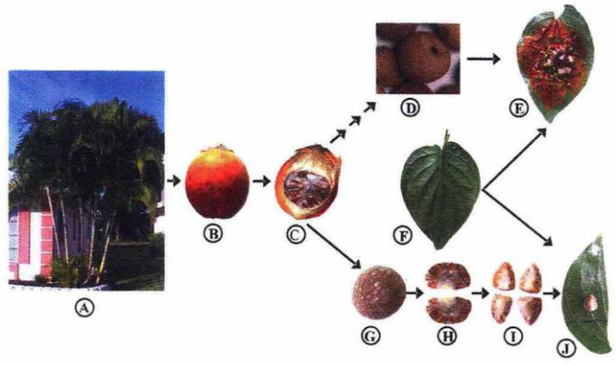

Fig. 15.1 Patterns of betel nut usage: *Areca catechu* L. palm trees in its natural habitat (**a**); a ripe betel fruit (**b**) and its cross section showing the betel nut (BN) encased within its fibrous shell (**c**). After appropriate curing, sun drying and removal of the shell, the dry and very hard variety of BN (**d**) is prepared, which is usually cut into small to very small pieces for mastication along with *Piper betle* leaf (**f**) as a betel quid (BQ) supplemented with a large variety of additives (**e**) (see text for details). BN is also masticated in its raw and wet form (**g**), which is usually cut into 4 pieces (**h** and **i**) and consumed as a simple BQ (**j**) comprising betel leaf (**f**), slacked lime and a piece of wet/raw variety of nut

component of the betel morsel. Chinese texts of 150 BCE, also mention BN as "*pin-lang*". In Persia (modern Iran), it is believed that around 30,000 shops sold BN in the capital town during the reign of Khosrau II, the King of Persia during 590–628 AD. There is also mention of use of BN in one or the other form in different parts of the world including South and South-East Asia, several Pacific islands, many regions of the former Soviet Union, parts of North America and Europe (Sharan, 1996). The use of BN is deeply ingrained in highly variable socio-cultural and religious practices across the globe (Warnakulasuriya, 2002). BN is believed to be used by both men and women across all age groups and social classes though in some societies the latter predominate (Warnakulasuriya, 2002). In old Indian scripts such as *Vagbhata* (fourth century), and *Bhavamista* (thirteenth century), BN has also been described as a "therapeutic agent". BN users report increased well-being and stamina, a soothing effect on the digestion, protection of the mouth and gums, and some euphoria. Its use was recommended in wide ranging human diseases and other disorders, which included vitiligo or leucoderma, leprosy, anemia, digestive disorders and infections, urinary and dental infections, and obesity. It has been suggested that BN chewing may confer protection against dental caries and other infections. In vitro evidence indicates that *Areca* tannins may have anti-microbial activity, which may contribute to the cariostatic properties of BN. Furthermore, betel stain, which coats the teeth of chewers, may act as a protective varnish (Trivedy et al., 2002). BN is also reported to have aphrodisiac property and has been recommended as a general stimulant. In China, it has been used as a vermifuge since the sixth century (Sharan, 1996). The BN is predominantly consumed in its dry form, which is usually a very hard nut (Fig. 15.1d). To make it easy to masticate or chew, the BN is cut into small to very small pieces (Fig. 15.1e). In contrast, people in several parts of the world,

including the whole of the north-eastern region of India, masticate the raw and wet form, which is relatively soft (Fig. 15.1 g). Hence, larger pieces of the nut are masticated (Fig. 15.1i). Aged people may masticate even powdered form of raw/wet or dry variety of BN.

Areca nut is normally harvested as unripe (green) or ripe (orange/red) fruit from the *Areca* palm (Fig. 15.1b, c). The *Areca* fruits may be sun dried for several weeks, fibrous shells removed and the hard, dry nuts are ready for use (Fig. 15.1d). Alternatively, the ripe *Areca* fruits are boiled for several hours in an aqueous solution containing the bark of the plant *Eugenia jambolana*, jaggery or brown sugar, and various edible oils, to "cure" it. The cured fruits are sun dried for several weeks, fibrous shell removed and very hard, brown nuts are ready for use (Fig. 15.1d). In contrast, ripe, partly ripe or unripe *Areca* fruits are freshly picked (Fig. 15.1b, c), fibrous shells removed and the relatively soft nuts are ready for masticated (Fig. 15.1 g). Occasionally, the fruits can be cured by burying them into moist pits for 1–2 weeks for fermentation (maturation) before deshelling and use. Such raw and wet variety of BN in the north-eastern part of India is locally called "*kwai*"or "*tambul*" (Fig. 15.1 g–i).

The BN is either consumed alone or with a wide variety of region and socio-culture specific additives as betel quid (BQ). In latter case, dry variety of BN is usually wrapped along with slaked lime (calcium oxide and calcium hydroxide or slacked lime) and catechu (*Acacia catechu*) without or with a host of additives, which may also include a variety of tobacco products, perfumes, stimulants, etc., in a piece of betel leaf (Fig. 15.1e, f). The raw/wet variety of BN is usually masticated with slaked lime wrapped in a betel leaf (Fig. 15.1e, j) and occasionally supplemented with chewing tobacco (IARC, 1985; Sharan, 1996; Warnakulasuriya, 2002). In India, most habitual chewers of BQ add tobacco, while in some countries, such as Papua New Guinea and China, tobacco is not added. Betel leaf is perishable and the preparation of BQ is somewhat complex (Fig. 15.1e). Hence, over the past three decades, commercial BQ substitutes, flavored and sweetened dry mixture of *Areca* nut, catechu and slaked lime with tobacco (*gutkha*) or without tobacco (*pan masala*), have become increasingly popular among habitual BN chewers (Nair et al., 2004).

15.1.1 Constituents of Betel Nut and Its Active Principles

The constituents of BN include carbohydrates, crude fiber, fats, polyphenols, alkaloids, tannins, proteins, ash and water. Trace amounts of fluorine, sapogenein, and free amino acids have also been reported in some forms. The relative amounts of these constituents are highly variable in dry or raw/wet variety of BN. Geographical and climatic conditions of growth of the *Areca* palm tree and the methods of curing BN also contribute to the observed variation in the constituents (Sharan, 1996). Table 15.1 shows the approximate content of different constituents of dry and raw/wet variety of BN. The raw and wet variety of BN is relatively rich in all constituents as compared to the dry variety. Notwithstanding these variations, the active

Table 15.1 Constituent of betel nut (BN): Approximate average percent constituent of dry and raw/wet varieties BN (IARC, 1985, 2004; Sharan, 1996)

Constituent	Dry variety (%)	Raw/wet variety (%)
Alkaloids		
Combined	0.25	0.35–0.49
Arecoline	0.15	0.18–0.24
Arecaidine	0.10	0.10–0.20
Others	Trace	0.14
Polyphenols	15	23
Tannins	18	22
Carbohydrates	25	30
Proteins	7.5	12
Fats	1.2	2.5
Fiber	15	18
Water	Low	High
Ash	Low	High

components of both forms of BN, which produce betel nut associated effects, are primarily the alkaloids, polyphenols, and tannins.

(a) *Alkaloids*: Alkaloids are reduced pyridines. BN contains primarily two alkaloids that are biologically highly relevant. Arecoline (1,2,4,5-tetrahydro-1-methyl-pyridinecarboxylic acid; molecular weight 155. 19 Da) is the most abundant alkaloid of BN followed by arecaidine (1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid; molecular weight 141. 17 Da). Other alkaloids such as, guvacine (methyl ester of arecaidine), guvacoline (methyl ester of guvacine) and arecolinidine are also present in small to very small or trace amounts (Table 15.1) (Sharan, 1996).

(b) *Polyphenols and tannins*: The main polyphenols of BN are catechin, flavanoids, flavan-3:4-diols, leucocyanidins and hexahydroxyflavans. When oxidized in the presence of lime, these give the characteristic red color to saliva, teeth and lips of BQ masticator. The predominant tannin of BN is gallotannic acid. In addition, minor amounts of gallic acid, D-catechol and phiobatannin are also present (Sharan, 1996).

(c) *Betel nut specific nitrosamines (BSNA)*: Numerous and highly complex nitrosamine derivatives are produced from different alkaloids of BN essentially by nitrosation of the alkaloid in the mouth and stomach, especially in acidic milieu, and in the presence of nitric oxide generated by bacterial action (Wary and Sharan, 1991; Boucher and Mannan, 2002). Figure 15.2 shows a typical and representative metabolic pathway of arecoline nitrosation and production of different derivatives. The major biologically relevant nitrosamines of arecoline, appropriately grouped as betel nut specific nitrosamines (BSNA), are N-(methylnitrosamino) propionaldehyde (NMPA), N-(methylnitrosamino) propionitrile (NMPN) and N-nitosoguvacoline. Of these, MNPA was reported to be the most potent BSNA on a molar basis effecting both survival and thiol content

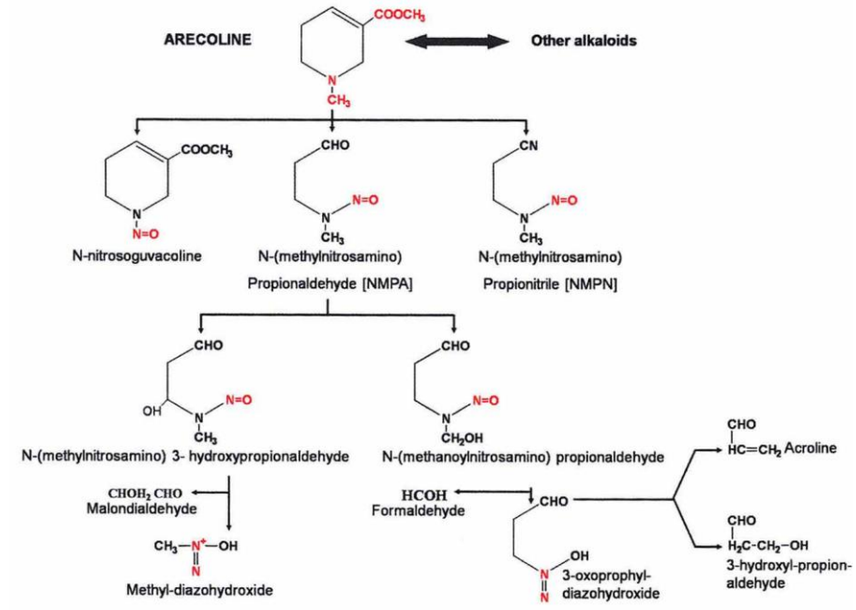

Fig. 15.2 Representative chemical pathway of metabolic activation of arecoline, the major carcinogenic alkaloid of BN. Different nitrosamine and their derivative are produced from the alkaloids, which have been called as betel nut specific nitrosamines (BSNA)

of cultured human buccal epithelial cells and causing significant formation of DNA single strand breaks (Sundqvist et al., 1989). It is proposed that NMPA may further generate N-(methylnitrosamino) 3-hydroxypropionaldehyde and N-(methanoylnitrosamino) propionaldehyde derivatives, each of which can potentially produce several diazohydroxide derivatives (see Fig. 15.2). Presence of most of these derivatives has been demonstrated in the saliva of BQ chewers (IARC, 1985; Nair et al., 1985).

(d) *Reactive oxygen species (ROS)*: Aqueous extracts of *Areca* nut and catechu were found to be capable of generating superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) at pH greater than 9.5 (Nair et al., 1987). While saliva was found to inhibit both $O_2^{\bullet -}$ and H_2O_2 formation from BQ ingredients, ROS are formed in the alkaline chewing mixture within the saliva of a chewer due to the addition of slaked lime (Stich and Anders, 1989).

15.1.2 General Effects of Betel Nut Consumption

BN is masticated or chewed for its psycho-stimulating effects (Norton, 1998). When BN is masticated, it usually produces mild psychoactive and cholinergic effects. Due to this, it is estimated that over 600 million individuals are habitual consumers of BN

in one form or the other world-wide (Sharan, 1996). Only three other "addictive" substances—nicotine, ethanol and caffeine, are reported to be more widely used by human beings (Norton, 1998). In north-east India, a raw/wet variety of BN called *kwai* or *tambul*, consumed with betel leaf and slaked lime, causes an immediate thermogenic physiological response lasting 2–3 min with significant perspiration on the forehead and reddening of ear pinnae (Sharan, 1996). There is copious production of blood-red saliva that stains oral structures. After years of chewing, the teeth may become red–brown to nearly black (Sharan, 1996; Boucher and Mannan, 2002). In vitro studies have demonstrated that *Areca* extracts containing arecoline inhibit growth and attachment of and protein synthesis in human cultured periodontal fibroblasts. These findings suggest that *Areca* may be cytotoxic to periodontal fibroblasts and may exacerbate preexisting periodontal disease as well as impair periodontal reattachment (Trivedy et al., 2002). The use of BQ was also found to be associated with the appearance of lichenoid lesions on the buccal mucosa and tongue, and betel chewer's mucosa, characterized by a brownish–red discoloration of the oral mucosa, often accompanied by encrustation of the affected mucosa with quid particles which are not easily removed, and with a tendency for desquamation and peeling (Trivedy et al., 2002).

Acute ill effects are also reported at high rates of usage of BN and include cardiac arrhythmia, exacerbation of asthma, acute psychosis and acute gut upset (Boucher and Mannan, 2002). Significant hyperglycemia was observed in male mice administered with BSNA, NMPN. In fact, a population study revealed increase in waist size and weight, taken as markers for hyperglycemia, in direct relation to *paan*, a type of Indian BQ, usage among Asians. These studies, thus, suggest that BN may be diabetogenic (Boucher and Mannan, 2002). BN chewing was found to be independently associated with increased urinary albumin excretion and albuminuria in Taiwanese male patients of type-2 diabetes (Tseng, 2006). BN alkaloids, especially arecoline, have anti-muscarinic effects on the smooth muscle. They are proposed to bind to GABA receptors in the brain, contributing to their psychoactive effects. BN chewing is thought to reduce the severity of symptoms in schizophrenia with reduction in both positive and negative symptoms. Withdrawal symptoms such as mood swings, anxiety, irritability, reduced concentration, sleep disturbance and craving were found to be associated with trying to quit the habit of BN chewing. These findings are regarded to be consistent with the existence of a dependence syndrome among regular users. In rare cases, *Areca* nut psychosis has been reported to occur in heavy users following abrupt cessation of the habit (IARC, 2004). One study of cases between 1988 and 1998 also reports toxicity of BN manifested in different individuals by tachycardia/palpitations, tachypnea/dyspnea, hypotension, sweating, vomiting, dizziness, chest discomfort, abdominal colic, nausea, numbness, coma, and acute myocardial infarction with its related manifestations (Deng et al., 2001).

15.1.3 Link Between Betel Nut and Carcinogenesis

Today, there is sufficient evidence that *Areca* nut or BN as well as BQ without or with tobacco is carcinogenic to humans (Sharan, 1996; IARC, 1985, 2004). BQ without tobacco causes oral cancer, while BQ with tobacco causes cancers of the oral cavity, pharynx and oesophagus (IARC, 2004). A causal association between tobacco and BQ chewing habits and oral mucosal diseases such as leukoplakia, oral submucous fibrosis (OSF) and oral cancer has been established, and heavy users have a significantly increased mortality rate. Oral cancer is the fifth most common cancer worldwide (Nair et al., 2004). Of the 390,000 oral and oro-pharyngeal cancers estimated to occur annually worldwide, 58% occur in south and south east Asia. In India, there is reported addition of 75,000–80,000 new cases of oral cancer each year and the incidence rates of cancers of the oral cavity in both males and females in all urban cancer registries are among the highest in the world. Time-trend analysis of cancers at all sites for the period 1990–1996 showed a decrease in cancers of the oral cavity in Indian population based registries, but an increase in the incidence of mouth cancer was reported among those aged < 50 years between 1983–1987 and 1995, consistent with the hypothesis of an increase in oral cancer among the young due to increased consumption of the alternative chewing products such as, *gutkha* and *pan masala* (Nair et al., 2004). In Taiwan, data on oral cavity cancer from the period between 1986 and 1997 indicated that those who chew BN belong to a high-risk group (Lin et al., 2005).

15.1.3.1 Induction of Pre-cancerous Lesions by Betel Nut

As an early sign of damage to the oral mucosa, chewers of BN or BQ with or without tobacco often develop clinically visible whitish (leukoplakia) or reddish (erythroplakia) lesions, which may or may not be accompanied by stiffening of the oral mucosa and OSF. These manifestations are well established precancerous lesions and are taken as early and important indicators of oral cancer risk to an individual. Some 2–12% of these lesions have been reported to turn malignant over several years. OSF, which is predominantly caused by the use of *Areca* nut, is a seriously debilitating and progressive disease marked by stiffening of the oral mucosa, development of fibrous bands and loss of elasticity of the mucosa, resulting in a progressive restriction of mouth opening. Flavonoids, catechins and tannins of BN cause collagen fibers to crosslink making them less susceptible to collagenase. This can cause increased fibrosis due to increased collagen production and decreased collagen breakdown. OSF is irreversible and persists even after cessation of the chewing habit, suggesting that components of the *Areca* nut initiate OSF and then affect gene expression in the fibroblasts, which then produce greater amounts of normal collagen (Nair et al., 2004). Considerable amounts of copper have been found in BN products. Copper salts significantly increase the production of collagen by oral fibroblasts in vitro supposedly by upregulation of activity of a copper-dependent enzyme, lysyl oxidase, which catalyses the cross linking of collagens and elastin and is implicated in the pathogenesis of OSF (Nair et al., 2004). In recent years, studies in India, China, south east Asia and South Africa, and on Asian migrants in the UK have shown a clear link between *Areca* nut chewing and OSF (Nair et al., 2004).

15.1.3.2 Betel Nut and Betel Nut Extracts in Carcinogenesis

An increased incidence of local tumors was observed in mice after subcutaneous injection of aqueous extracts of BQ without tobacco. Local tumors were produced in mice and local mesenchymal tumors in rats following subcutaneous injection of aqueous extracts of betel nut (AEBN). In hamsters, administration of *Areca* nut and application of its aqueous or dimethyl sulphoxide extracts to the cheek-pouch mucosa resulted in squamous cell carcinomas (SCC) of the cheek pouch and carcinomas of the fore-stomach (IARC, 1985). While BQ has various components (IARC, 1985; Sharan, 1996; Warnakulasuriya, 2002), a study on Syrian hamsters revealed that BN fiber and cold aqueous extract are the major components of BQ that may promote carcinogenesis in the hamster buccal pouch, leading to tumor formation. AEBN has been shown to induce conformational changes in mouse liver high mobility group (HMG) proteins similar to that induced by a hepatocarcinogen, diethylnitrosamine (DEN), leading to the development of preneoplastic nodules in the liver (Pariat and Sharan, 1998a, b). The post-translational modification of proteins such as, poly-ADP-ribosylation of HMG (Pariat et al., 1999; Pariat and Sharan, 2002) and histone (Saikia et al., 1998, 1999a, b) proteins was also strongly affected by exposure to BN resulting in alterations in chromatin organization.

(a) *Cytotoxicity: Areca* nut extract was found to decrease cell survival, vital dye accumulation and membrane integrity of cultured human buccal epithelial cells in a dose-dependent manner. BN also caused formation of both DNA single strand breaks and DNA protein cross links (Wary and Sharan, 1988; Sundqvist et al., 1989, Wary and Sharan, 1991). Different extracts of BN such as, AEBN, acetic acid extract (AAEBN), HCl extract (HEBN) and ethanol extract (EEBN) as well as arecoline showed different extents of cytostatic and cytotoxic effects on Hep2 cells in vitro, with arecoline, HEBN and EEBN being the most potent (Sharan and Wary, 1992). Cultured normal human oral keratinocytes (NHOK) exposed to ripe BN extract also showed significant decrease in population doubling, increase in senescence, cell cycle arrest at G_1/S phase and decrease in cell proliferation (Lu et al., 2006). Hamsters fed with powdered diet containing BN or BQ showed significant decrease in the survival rate, body weight, and hyperkeratosis and acanthosis of cheek pouch indicating that BN and BQ components may induce alterations in proliferation and differentiation of oral epithelial cells (Chiang et al., 2004).

(b) *Genotoxicity:* BQ and its components were found to be genotoxic. Interestingly, they also stimulated cell proliferation making the observed biological effects very complex. For instance, while the extracts of BN and inflorescence of *Piper betle* (IPB) induced DNA strand break, the extracts of BN, IPB, the BN polyphenol (+/−)catechin and arecoline decreased cell survival and proliferation. On the other hand, another component of BQ, the aqueous extract of lime, was found to increase cell proliferation (Jeng et al., 1994). AEBN was found to reduce glutathione synthetase (GSH) levels, induce chromosomal aberrations (CA) and delay cell kinetics in mouse bone marrow cells with the induction of sister chromatid exchange (SCE) probably involving TP53 dependant changes in cell proliferation (Kumpawat et al., 2003). Ethyl acetate and *n*-butanol extracts of BN as well as betel leaf are reported to induce CA in human lymphocytes and Chinese hamster ovary (CHO) cells (IARC, 1985). All components of BQ have been shown to individually enhance chromatid breaks and exchanges in the range of 12–37% in human cells in vitro. Frequency of SCE was elevated in mouse bone marrow cells when mice were exposed to the AEBN and its tannin (Panigrahi and Rao, 1989). AEBN also induced DNA strand breaks and enhanced cell proliferation in mouse kidney cells in vitro (Wary and Sharan, 1988). A study revealed that OSF was largely associated with BN and the exfoliated oral mucosal cells of such patients had significantly higher numbers of micronucleated cells. The patients also exhibited increased SCE in circulating lymphocytes indicating that the carcinogenic agents in BN produce damage not only in target tissue but also in other host cells such as circulating lymphocytes (Desai et al., 1996).

(c) *Immunotoxicity:* Aqueous extracts of raw *Areca* nut without husk as well as with husk were found to inhibit the phagocytic activity of human neutrophils in a dose dependent manner (Hung et al., 2005). BQ also influenced cytokine production of peripheral blood mononuclear cell. The mononuclear cells of persons suffering from SCC, with a long history of BQ chewing, produced lower levels of TGF-β, TNF-α and IFN-γ in comparison to normal persons (Hsu et al., 2001). This was indicative of compromised immune system under the influence of BQ or BN chewing.

(d) *Mutagenicity:* Aqueous extracts of BQ without tobacco induced mutations in *Salmonella typhimurium* but not in Chinese hamster V79 cells. BQ also did not induce any significant micronuclei in Swiss albino mice (IARC, 1985). AEBN, on the other hand, induced mutations in *Salmonella typhimurium* and in Chinese hamster V79 cells besides inducing gene conversion in *Saccharomyces cerevisiae* as well as CA in CHO cells. It also induced micronuclei in bone marrow cells of Swiss albino mice while BN tannin fraction induced gene conversion in *Saccharomyces cerevisiae* (IARC, 1985). Arecoline, AEBN, AAEBN, HEBN and EEBN induced variable levels of dose dependent unscheduled DNA synthesis (UDS) in Hep2 cells in vitro (Sharan and Wary, 1992; Sharan, 1996). Ames test using *Salmonella typhimurium* strain TA 1535 revealed that arecoline, AEBN and HEBN were weak mutagens while AAEBN and EEBN were strong mutagens suggesting that the mutagenic potential of alkaloids (arecoline) could be significantly enhanced by other constituents of BN (Sharan, 1994; Balachandran and Sharan, 1995; Sharan, 1996). Exposure to BN extracts was found to induce mutations at the *hypoxanthine phosphoribosyltransferase* (*HPRT*) locus in human keratinocytes (HaCaT cells), which also increased frequency of appearance of micronuclei, intracellular levels of reactive oxygen species and 8-hydroxyguanosine in the cells suggesting that stress caused by long-term BN extract exposure enhanced oxidative stress and genetic damage in HaKaT cells (Lai and Lee, 2006). When aqueous extracts of different brands of *pan masala* and scented BN or *supari* were tested for mutagenicity by the

Salmonella typhimurium assay using tester strains TA98 and TA100 their mutagenic effects were found to be similar to that produced by BN extracts (Polasa et al., 1993). A study involving patients of head and neck cancer suggested that BQ chewing may increase mitochondrial DNA (mtDNA) mutation in human oral tissues and that accumulation of mtDNA deletions and subsequent cytoplasmic segregation of these mutations during cell division could be important contributors to the early phase of oral carcinogenesis (Lee et al., 2001).

(e) *Teratogenesis:* Aqueous extracts of dry as well as raw/wet varieties of BN were reported to be fetotoxic in Swiss albino mice leading to death, enhanced resorption and reduced weight of fetuses. Other abnormalities such as hematomas, curved tails, abnormal ribs and delay in skeletal maturity have also been reported (Sinha and Rao, 1985a).

15.1.3.3 Betel Nut Alkaloids in Carcinogenesis

Alkaloids of BN are suspected to be its main carcinogenic constituent (IARC, 1985, 2004; Sharan, 1996; Norton, 1998; Jeng et al., 2001; Trivedy et al., 2002). Early studies found that the application of arecaidine to the oral mucosa of experimental animals failed to have any carcinogenic effects unless it was supplemented with a known promoter such as, croton oil (Trivedy et al., 2002). Arecoline given by gavage produced lung adenocarcinomas, stomach SCC and liver haemangiomas in male mice (IARC, 2004). Cheek-pouch application of arecoline following application of slaked lime produced an esophageal papilloma in female hamsters, while local application of arecaidine to the cheek pouch did not produce tumors in male hamsters (IARC, 2004). To explain the variable observation, it is proposed that the alkaloids first required metabolic activation via nitrosation to develop its carcinogenicity (Wary and Sharan, 1991). In rats, the major metabolic pathway of arecoline activation is via de-esterification and production via conjugated mercapturic acid. In vitro data suggest that arecoline is metabolized by carboxylesterase (EC 3.1.1.1) in mouse liver and kidney. Male Swiss albino mice fed *Areca* nut powder or arecoline showed enhanced levels of the hepatic cytochrome P450 and b_5 and decreased levels of hepatic GSH (IARC, 2004). Exposure of Swiss albino mice to arecoline was found to lower poly-ADP-ribosylation of most cellular and histone proteins and induce relaxation of chromatin, thereby allowing the N-nitrosamines of arecoline easy access to genomic DNA for interaction, while the absence of PADPR induced repair may favor the accumulation of DNA damage (Saikia et al., 1999b).

(a) *Cytotoxicity*: Arecoline was found to inhibit cell attachment, cell spreading and cell migration in a dose dependent manner in cultured human gingival fibroblasts (HGF) (Jeng et al., 1996). In Hep2 cells in vitro, arecoline inhibited both DNA and protein syntheses in a dose dependent manner, which ultimately resulted in cytostatic effect on cell division (Wary and Sharan, 1991). Crude alkaloid extracts of green *Areca* fruit consumed in Taiwan and arecoline were found to be mutagenic in *Salmonella typhimurium* TA100, and

N-Nitrosoguvacoline (NG) was weakly mutagenic in TA98 and TA100, with the formation of NG being favored at neutral pH (Wang and Peng, 1996). Arecoline alone was also only weakly mutagenic (Balachandran and Sharan, 1995). In vitro studies have shown that arecoline and arecaidine may stimulate cultured fibroblasts to proliferate and synthesize collagen, an important step in development of OSF. However, subsequent in vitro studies have failed to show similar effects of arecoline on cultured OSF fibroblast. It has also been shown that arecoline inhibited collagen synthesis and fibroblast proliferation in vitro, indicating the cytotoxic properties of arecoline. The disparity of results from in vitro studies might be indicative of other agents, in addition to arecoline, being important in the pathogenesis of OSF (Trivedy et al., 2002). The cytotoxicity of arecoline on the oral mucosal fibroblast (OMF) or on Hep2 cells was found to be associated with cellular GSH levels and esterase activities on one hand (Jeng et al., 1999), and the agents that facilitate metabolic activation and nitrosation of alkaloids, on the other (Wary and Sharan, 1991). In fact, GSH depletion and reduction of glutathione *S*-transferase activity have been demonstrated in cultured human oral keratinocytes and in fibroblasts treated with arecoline (IARC, 2004). Arecoline was also reported to be cytotoxic to human buccal fibroblasts in a dose dependent manner wherein the cellular glutathione-S-transferase (GST) activity was downregulated in a dose dependent manner without increase in lipid peroxidation. Addition of extracellular nicotine acted synergistically on the arecoline-induced cytotoxicity, indicating that arecoline may render human OMF more vulnerable to other reactive agents in cigarettes via GST reduction. These observations could explain why patients who practice the combined habit of BQ chewing and cigarette smoking are at greater risk of contracting oral cancer (Chang et al., 2001a).

Global gene expression profiling in HGF exposed to arecoline revealed that four genes related to maintenance of genome stability and DNA repair were repressed by arecoline (Chiang et al., 2007). They are *FANCG*, also known as *XRCC*9 (tumor suppressor capable of correcting CA), *CHAF*1 and *CHAF*2 (encoding chromatin assembly factor I, CAF1), and *BRCA*1 (breast cancer susceptibility gene implicated in DNA damage response and DNA repair). Among them, at least the *BRCA*1 response was dose dependent. *COX-2/PTGS*2, which are involved in cancer initiation and progression, were over expressed in HGF cells. *HSP*4*A*1 and *DNAAJA*1, which belong to the *HSP*70 family of stress induced proteins, and GDF15/MIC-1 were also upregulated by arecoline in a dose dependent manner (Chiang et al., 2007).

(b) *Genotoxicity*: Arecoline was found to induce mutations in *Salmonella typhimurium* and Chinese hamster V79 cells, and CA in CHO cells. It also induced micronuclei, CA and SCE in bone marrow cells of Swiss albino mice (IARC, 1985; Deb and Chatterjee, 1998). However, upon withdrawal of arecoline exposure regime from Hep2 cells in vitro the inhibited DNA synthetic index fully recovered (Wary and Sharan, 1991) suggesting existence of weak interaction between BN genotoxin and DNA. Arecaidine induced mutations in *Salmonella typhimurium* and Chinese hamster V79 cells. It also induced SCE

but not micronuclei in bone marrow cells of Swiss albino mice (IARC, 1985). This arecoline induced DNA damage was found to be influenced by endogenous GSH levels with the frequency of CA and SCE increasing when arecoline was given to mice treated with buthionine sulfoximine (BSO), a GSH depleting agent (Lu et al., 2006).

(c) *Immunotoxicity*: Arecoline was found to cause inhibition of both humoral and cell-mediated immune responses in mice (IARC, 2004). It is reported to interfere with the immune system by targeting the muscarinic acetylcholine receptors of the non-neuronal cholinergic system (Wen et al., 2006). Arecoline was also found to inhibit the phagocytic activity of human neutrophils (Hung et al., 2005).

(d) *Cell-cycle alterations*: Arecoline inhibited growth of human KB epithelial cells in dose- and time dependent manners by causing cell cycle arrest in late-S and G2/M phases due to induction of cyclin Bl, Wee 1, and phosphorylated cdc2 proteins and inhibition of p21 protein expression in KB cancer cells. In primary human gingival keratinocyes (HGK) arecoline effect was mediated differently. In this case, arecoline induced p21 but inhibited cdc2 and cyclin B1 proteins. This clearly suggests that differential regulation of S and/or G2/M cell cycle related proteins in the HGK and KB cells play crucial roles in different stages of BQ mediated carcinogenesis (Lee et al., 2006). Arecoline, which was cytotoxic to HGF cells due to depletion of intracellular thiols and inhibition of mitochondrial activity, induced cell cycle arrest in HGF cells at G2/M phase in a dose dependent manner (Chang et al., 2001b).

(e) *Teratogenicity*: Arecoline has been reported to induce abnormality in the shape of sperm heads and unscheduled DNA synthesis (UDS) in the early spermatid stages of Swiss albino mice (Sinha and Rao, 1985b). It also induced micronuclei formation in fetal mouse blood after transplacental exposure to BN (Sinha and Rao, 1985c). Arecoline caused general developmental retardation of zebra fish embryos predominantly due to a general cytotoxic effect induced by depletion of intracellular thiols (Chang et al., 2001c). Arecoline hydrobromide has been reported to have teratogenic effects on developing chick embryos leading to embryo mortality, retarded development of fetuses and other abnormalities. The abnormalities included reduced body size, scanty feathering, general edema with light body color, shortened lower beak, clubfoot, missing or unossified rib and shortening of long bones (Paul et al., 1999).

15.1.4 Betel Nut and Tumor Suppressor Genes TP53, BRCA1 *and* BRCA2

Tumor suppressor genes are critical in carcinogenesis because loss of their function(s) results in promotion of malignancy (Kinzler and Vogelstein, 1997). Prominent among them is *TP*53 gene encoding a 393-amino acid residue long p53 protein, which is maintained at low cellular level in normal cells due to MDM2

mediated rapid turnover (Lane, 1992; Levine, 1997). Cells exposed to carcinogen or other stresses rapidly accumulate p53 due to its stabilization and/or mutation. Mutated or stabilized p53 induces cell cycle arrest at G1/S or G2 checkpoints. The quiescent cells are now in a position to repair the damage caused by the carcinogen or other stress factors and come out of it. Thus, TP53 functions as a "gatekeeper" tumor suppressor. The breast cancer susceptibility genes *BRCA*1 and *BRCA*2 are other two tumor suppressor genes relevant to human carcinogenesis. Both Brca1 and Brca2 proteins are functionally grouped as "caretakers" as they are involved with repair of DNA breaks, especially the critical double stranded breaks (DBS), via homologous recombination (HR) repair pathway in association with RAD family and other proteins.

Consistent with projected functions of *TP*53, *BRCA*1 and *BRCA*2 tumor suppressor genes, mutation or alteration in expression or both is expected in these tumor suppressor genes/proteins during carcinogenesis. Indeed, *TP*53 gene, one of the most extensively studied tumor suppressor genes, is known to be mutated in a variety of human and experimental animal cancers. Similarly, change in cellular level of p53 protein is also known to occur. Accumulation of p53 protein or its stabilization is an important indicator of the presence of mutant p53 protein (Hollstein et al., 1991, Harris and Hollstein, 1993). However, reports pertaining to *TP*53 mutation status of cancers associated with BN chewing have been widely contradicting. A study of Sri Lankan subjects with histologically confirmed oral squamous cell carcinoma (OSCC) and the habit of BN chewing with tobacco revealed low expression of p53 protein (Ranasinghe et al., 1993). A similar study in BN and tobacco associated OSCC from Southern India showed nuclear TP53 staining and TP53 expression indicating that carcinogens derived from tobacco and BN chewing may induce TP53 mutations (Kuttan et al., 1995). BQ chewers in Taiwan exhibited significantly higher incidence of *TP*53 gene mutations than non-chewers in esophageal squamous cell carcinoma (ESCC). The A:T → G:C transition and G:C → T:A transversion were the prevalent spectra of *TP*53 gene mutations and alcohol consumption could enhance this peculiar spectrum of *TP*53 mutation in ESCC suggesting that *TP*53 might be an important molecular target of BQ carcinogens in the development of ESCC in Taiwanese (Goan et al., 2005). Another study on patients of OSCC in Taiwan revealed that G:C → A:T transitions were the predominant mutations in the *TP*53 gene associated with BQ and tobacco use (Hsieh et al., 2001). Mutations in the *TP*53 gene were also frequent in OSCC specimens from Sri Lanka obtained from BQ chewers. They exhibited point, small deletion and addition type of mutations mainly clustered in exon 5 of the *TP*53 gene. These results indicate that exon 5 of the *TP*53 gene could be one of the specific targets for some BQ ingredients, and BQ chewing may be a critical environmental factor in the development of OSCC (Chiba et al., 1998). A study of potentially malignant oral lesions (leukoplakia) and OSCC associated with BQ consumption in northern India revealed a good correlation between TP53 missense mutations, p53 antibodies and p53 protein accumulation in matched potentially malignant and malignant oral lesions (Ralhan et al., 2001). Alternatively, incidence of *TP*53 mutations was reported to be infrequent or absent in oral premalignant lesions and OSCC in subjects chewing BQ with

tobacco (Kannan et al., 1999) and without tobacco (Thomas et al., 1994; IARC, 2004). Mutations in both BRCA genes are known to be prevalent in familial as well as sporadic breast cancers (Rajan et al., 1996, Nadeau et al., 2000). However, not much is known about the status of these two important tumor suppressor proteins in BN associated carcinogenesis in mice or men.

We have made a systematic effort to study the effect of long term and transgenerational exposure of Swiss albino mice to AEBN on expression of TP53, Brca1 and Brca2 proteins as well induction of mutation in exons 5 and 7 of the *TP*53 gene and exon 11 of the *Brca*1 gene. Chronic exposure to AEBN in drinking water led to an upregulation of p53 protein in liver, spleen and peripheral blood lymphocytes (PBL) of exposed parental (P) generation mice from 2 weeks onwards reaching a maximum (2.5 folds of the age-matched control) after 6 weeks of exposure in the liver and spleen and 4 weeks of exposure in PBL (Fig. 15.3, panel A). Subsequently, the level of p53 protein declined gradually reaching control level after 16 weeks of exposure concomitant with the appearance of pre-neoplastic nodules in the liver (Fig. 15.3, panel A). After 24 weeks of exposure p53 protein was below control

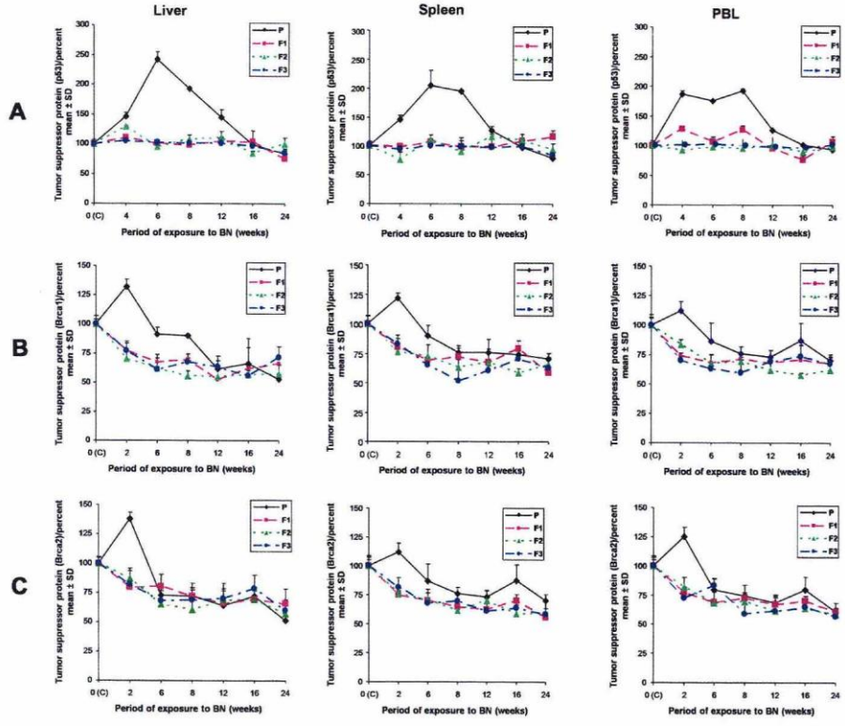

Fig. 15.3 Graphs showing cellular levels of three tumor suppressor proteins, p53 (**a**), Brca1 (**b**) and Brca2 (**c**), in liver, spleen and peripheral blood lymphocytes (PBL) of mice chronically and transgenerationally exposed to aqueous extract of betel nut (AEBN) in drinking water from parental (P) generation to F1 through F3 generations of mice

level, and the pre-neoplastic nodules were well developed. The expression of Brca1 (Fig. 15.3, panel B) and Brca2 (Fig. 15.3, panel C) proteins showed immediate elevation in liver, spleen and PBL after 2 weeks of exposure followed by a decline to 60% of that of age-matched control after 16 weeks of exposure and 50% after 24 weeks of exposure. No mutation in exons 5 and 7 of the *TP*53 gene (GenBank accession # EF570972 and EF634061) (Choudhury and Sharan, 2009) and exon 11 of the *Brca*1 gene (Choudhury and Sharan, 2010) were detected. Transmission electron microscope (TEM) study of the liver pre-neoplastic nodules after 24 weeks of exposure revealed a large number of binucleated cells with enlarged and abnormally shaped nuclei (Fig. 15.4b) as compared to the controls (Fig. 15.4a). Disruption of nuclear membrane as well as chromatin condensation and marginalization were also observed in a significant number of nuclei (Fig. 15.4c, d). Damage to mitochondria was most noticeable. The size of normal mitochondria (Fig. 15.4e) was significantly reduced (Fig. 15.4f) in all cases showing shrinkage. This was also accompanied with membrane disruptions (Fig. 15.4c, f; arrow head). The rough endoplasmic reticulum membrane organization (Fig. 15.4 h) was also severely damaged (Fig. 15.4i; arrow head).

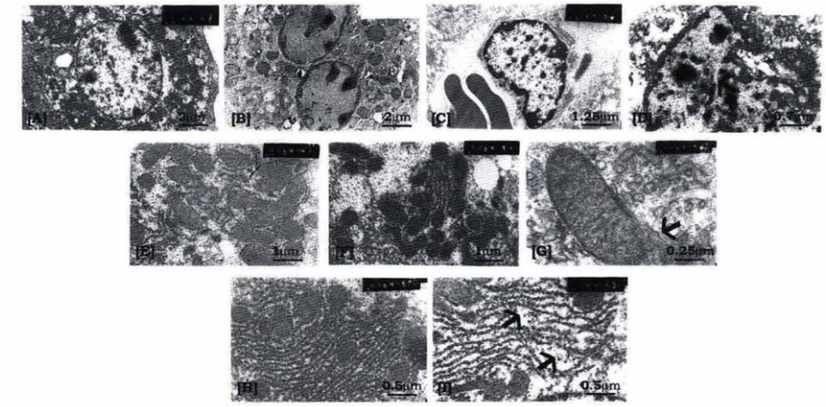

Fig. 15.4 Transmission electron micrographs of normal and transformed liver sections of mice exposed to aqueous extract of betel nut (AEBN) in drinking water. A normal liver cell with regular nucleus (**a**), which upon exposure to AEBN often showed binucleated cells (**b**), deformed nucleus (**c**) and/or nucleus with condensed and marginalized chromatin (**d**). The regular mitochondria of a normal cell (**e**), exhibited shrinkage and reduction in size upon exposure to AEBN (**f**) often accompanied with disrupted mitochondrial membrane (**g**). The normal arrangement of membrane in the endoplasmic reticulum (ER) (**h**) also exhibited pronounced disruptions (**i**)

Extensive damage of the mitochondrial membrane is a pro-apoptotic signal and extensive disruption of the ER could lead to calcium release from the ER lumen, which can potentially trigger ER-stress induced apoptosis. Thus, chronic exposure to AEBN caused serious molecular and metabolic damage to cells characterized by enlarged nuclei, high frequency of abnormally shaped nuclei, chromatin condensation and marginalization, and damaged membrane (Fig. 15.4) along with

downregulated p53, Brca1 and Brca2 proteins (Fig. 15.3). After 16 weeks of chronic exposure to AEBN, an inability to upregulate TP53 beyond control level, combined with compromised DNA repair due to downregulation of Brca1 and Brca2, are sufficient to allow progression of hepatocarcinogenesis.

The effect of prenatal and transgenerational chronic exposure to AEBN has been followed up to F3 generation by breeding. In striking contrast to the P generation, the liver, spleen and PBL of AEBN exposed F1, F2 and F3 generation mice exhibited invariant expression of p53 protein in comparison to age matched controls throughout the period of exposure (Fig. 15.3a). Similarly, the expression of Brca1 (Fig. 15.3b) and Brca2 (Fig. 15.3c) proteins progressively declined to approximately 80% that of age matched controls only after 2 weeks of exposure in all the three tissues examined. Thus, while the P generation mice exhibited an induction of the tumor suppressive functions of the p53, Brca1 and Brca2 proteins during the initial periods of AEBN exposure, the transgenerationally exposed mice failed to induce these tumor suppressors (Fig. 15.3). Concomitantly, a significant advancement in the appearance of liver pre-neoplastic nodules was observed with each subsequent generation suggesting progressive enhancement of transmission of carcinogenic risk due to exposure to BN constituents (Table 15.2). Abnormalities, which were not observed in P generation mice, also developed in the transgenerationally exposed mice. Though statistically insignificant up to F3 generation, these abnormalities included enlargement of lymph nodes of the neck, development of protuberant pus-filled sacs, necrosis of the liver and development of solid tumors. No mutations in exons 5 and 7 of the *TP*53 gene were observed in the liver nodules as well as solid tumors of even the transgenerationally exposed mice. Thus, while inactivation of the TP53 gene apparently plays a crucial role in BN associated cancer in mice, the inactivation is not ubiquitously through *TP*53 mutation and other routes of inactivation require to be investigated. One possible alternative mechanism for TP53 inactivation in BN carcinogenesis may be over-expression of MDM2 protein as has been shown in OSCC (Shwe et al., 2001). In contrast BN induced solid tumors in Swiss albino mice carried a G → C (codon 156; –TGT– → –TCT–) transversion mutation in exon 11 of the *Brca*1 gene (Fig. 15.5). Exon 11 of the *Brca*1 gene encodes the two nuclear localization motifs and a region of the gene believed to be essential for binding of RAD51 (Cressman et al., 1999). Mutation in exon 11 would, therefore, disrupt normal functioning of the *Brca*1 gene leading to DNA repair defects, which could be pivotal for the development of the solid tumors. Thus, AEBN can possibly

Table 15.2 Estimated transgenerational cancer transmission risk in mice from BN (Choudhury and Sharan, 2009)

Chronic AEBN administration in drinking water to mice	Appearance of pre-neoplastic nodules on liver
P generation	16 weeks
F1 generation	8 weeks
F2 generation	6 weeks
F3 generation	4 weeks

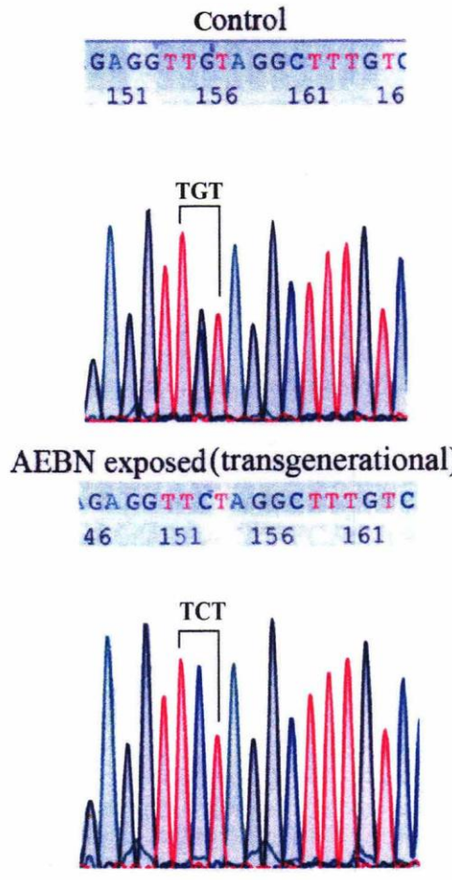

Fig. 15.5 Part of the nucleotide sequence chromatograms of PCR amplicons representing exon 11 of *Brca*1 gene of control (*top*) and F1 generation of AEBN exposed (*bottom*) mice liver. It shows induction of a G → C transversion type point mutation

lead to transgenerational transmission of carcinogenic risk in Swiss Albino mice by compromising the functions of the tumor suppressor genes *TP*53, *BRCA*1 and *BRCA*2 via different mechanisms.

15.1.5 Betel Nut Polyphenol and Tannins in Carcinogenesis

Toxicity studies relating to BN specific polyphenols and tannins are not conclusive with both carcinogenic and anti-carcinogenic effects being reported. It is reported that ROS produced during auto-oxidation of BN polyphenols in the BQ chewer's saliva are crucial in the initiation and promotion of oral cancer (Jeng et al., 2001). Incidences of certain cancers, such as esophageal cancer, have been reported to be related to consumption of tannins-rich foods such as BN suggesting that tannins

might be carcinogenic. However, other reports indicated that the carcinogenic activity of tannins might be related to components associated with tannins rather than tannins themselves (Chung et al., 1998).

15.1.6 Betel Nut and Human Genetic Susceptibility to Oral Cancer

Exposure to BN carcinogens, particularly the alkaloids, enhances the risk of cancer in BN or BQ chewers in general. However, correlation between prevalence of cancer in human populations in different parts of the world and habit of BN/BQ mastication is not absolute. This suggests that the genetic makeup of the masticator has its own influence on the ultimate manifestation of BN induced cancer. It is becoming obvious that the interplay between the genetic constitution and the environmental factor(s), determine the final risk of human oral cancer following exposure to BN or BQ alone or in combination with additives, including tobacco. Mere exposure to BN or BQ does not commit the chewer to cancer. For any given level of exposure to BN carcinogen, only a proportion of exposed individuals will develop cancer, indicating the prevalence of inter-individual differences in susceptibility (Spitz and Bondy, 1993). Individual susceptibility to cancer may result from several factors including (a) differences in metabolism, (b) status of DNA repair pathways and related genes, (c) patterns of expression of proto-oncogenes and tumor suppressor genes, and (d) nutritional status of the masticator, etc. Variations in an individual's metabolic phenotype, i.e., phenotypic polymorphism, have also been detected in a variety of enzymes involved in activation and detoxification of chemical carcinogens. It is becoming clearer now that different phenotypic and/or metabolic variations stem from genetic polymorphisms prevalent in different population groups (Bartsch and Hietanen, 1996). A number of genetic polymorphisms have been identified, which seem to be associated with risk of BN induced oral cancer in human sub-populations. Table 15.3 depicts the up to date list of polymorphisms observed in BN exposed human sub-populations with manifestation of oral cancer.

15.1.7 Possible Mechanism of Betel Nut Induced Carcinogenesis

BN is a natural plant product characterized by a very complex and highly variable mixture of different biochemical and nutraceutical constituents (Table 15.1). Some of these are recognized as potent carcinogens (e.g., alkaloids, polyphenols, tannins, etc.). However, many others, especially those present in small to trace amounts, have largely unknown biological functions. As with nutraceuticals, it is anticipated that in a complex cellular environment some of these may function as mediators, some as modulators, affecters, promoters, and/or inhibitors, etc. eliciting a variety of biological effects and responses. The highly variable constituents of BN should also chemically and otherwise interact differently with different biomolecules. The

Table 15.3 Genetic polymorphism and susceptibility to oral cancer in humans

No.	Gene/region	Polymorphism	Effect	Population group	References
1	Matrix metallo-proteinase-9 (MMP9) promoter	1562 C-to-T polymorphism	MMP-9-1562 C>T polymorphism—enhanced OSCC risk in young male BN chewers	Taiwanese	Tu et al. (2007)
2	Matrix metallo-proteinase-3 (MMP3) promoter	Insertion/deletion (-1171 5A→>6A) polymorphisms	5A genotype polymorphism—enhanced risk of OSF but not OSCC among male BN users	Asian	Tu et al. (2006)
3	NFKB1 promoter	Insertion (ins)/deletion (del) polymorphism (-94 ins/del ATTG) in NFKB1 promoter.	NFKB1 ins and HO-1 L allelotypes—significantly enhanced risks for different subsets of OSCC in male BN chewers	Asian	Lin et al. (2006)
4	DNA repair genes *XRCC1* and *XPD*	Polymorphisms Arg194Trp, Arg280His, and Arg399Gln of the XRCC1 gene and Lys751Gln of the XPD gene	Variant allele of XRCC1 399 codon and XPD—enhanced risk of oral cancer among BQ chewers and smokers	South Indian	Ramachandran et al. (2006)
5	Heme oxygenase-1 (*HMOX1*)	Polymorphisms in a (GT)n microsatellite repeat in HMOX1 promoter in short (S), medium (M) and long (L) alleles	Longer (GT)n repeat allele L—higher risk of BN related OSCC; (GT)n repeat allele S—may be protective for OSCC	Asian	Chang et al. (2004)
6	Cytochrome gene *CYP2A6*	*CYP2A6*4C* mutation-gene deletion type of polymorphism	Deficient *CYP2A6* activity due to deletion—reduced risk of oral cancer risk in BQ chewers	Sri Lankan	Topcu et al. (2002)
7	Cytochrome gene *CYP1A1*	*CYP1A1 A/G* genotype (Ile/Val) and *G/G* genotype (Val/Val) in exon 7	*CYP1A1* exon 7 containing *G* allele—enhanced risk for OSCC and oral precancerous lesion (OPL) in BN chewer and smoker	Chinese	Kao et al. (2002)

Table 15.3 (continued)

No.	Gene/region	Polymorphism	Effect	Population group	References
8	Collagen related genes: Collagen 1A1 and 1A2 (COL1A1 and COL1A2), Collagenase-1 (MMP1), transforming growth factor β1 (TGFB1), Lysyl oxidase (LOX), and Cystatin C (CST3)	Polymorphisms of six collagen related genes, COL1A1, COL1A2, MMP1, TGFB1, LOX, and CST3	Multigenic mechanisms involving the collagen related genes enhance susceptibility to OSF among BQ chewers	Taiwanese	Chiu et al. (2002)
9	Tumor necrosis factor-α (TNFA)	Bi-allelic promoter region (-308) polymorphism on the TNFA gene	The high production allele, TNF2—significantly lower among individuals with OSF	Taiwanese	Chiu et al. (2001)
10	Glutathione-S-transferase genes GSTM1 and GSTT1	GSTM1 and GSTT1 null genotypes (GSTM1*2 and GSTT1*2)	Null genotypes of either or both GSTM1 and GSTT1—enhanced risk of development of leukoplakia following exposure to tobacco with or without BQ	South Indian	Nair et al. (1999)
11		Genetic polymorphism of GSTM1 and GSTT1	Homozygous deletion of GSTM1 gene—enhanced risk for oral cancer, which is further compounded by exposure to cigarette smoke, alcohol, and BQ	Thai	Kietthubthew et al. (2001)

situation is further complicated by the fact that a host of region, culture and society specific additives, notably different types of chewing tobacco, are invariably added to BN preparation by a traditional masticator (Fig. 15.1). Therefore, it is only expected that the mechanism of BN induced carcinogenesis would also be highly variable and complex. Nonetheless, certain conclusions can be drawn from the wealth of knowledge available to us (see Fig. 15.6). The overall perception is that alkaloids are the main carcinogenic constituents of BN. Polyphenols and tannins may also contribute positively to carcinogenic potency of the alkaloids. It

Fig. 15.6 Schematic diagram of major metabolic events and milestones in the pathway of betel nut induced carcinogenesis (see text for details)

is now accepted that alkaloids, the primary suspected carcinogen of BN, should first undergo metabolic activation and nitrosation to produce the ultimate carcinogenic derivatives together called BSNA (Fig. 15.2). This may be achieved by de-esterification using carboxylesterase and may also involve cytochrome P450, b_5 and GSH besides other metabolites. The activated or ultimate carcinogen(s) acquires capability of interaction with target biomolecules. The interaction of BSNA or their activated derivatives with cellular targets forming adducts of different kinds may be the beginning of carcinogenesis (Chen et al., 1999; Liu et al., 2004). Using different physic-chemical methods evidence of existence of BSNA adducts has been amply demonstrated (IARC, 1985). Recently, formation of DNA adducts by AEBN and its consequences has been directly shown on a plasmid DNA construct, pMTa4 (Bhattacharjee and Sharan, 2008). Using the plasmid model in vitro and in vivo it has been shown that under chronic exposure condition up to one BN specific adduct could be formed every 3 nucleotides. In other words, up to 3 adducts can potentially be formed per helical turn of DNA double helix. These adducts were essentially unstable and dissociated from DNA in about 24 h in line with known weak, non-covalent and reversible nature of interaction of BSNA or their metabolites with DNA (Wary and Sharan, 1991; Sharan, 1996). However, we have also discovered that the BN adducts became stable in the presence of trace amounts of monovalent cations, Na^+ and K^+ (Bhattacharjee and Sharan, 2008). Since physiological cellular concentrations of these monovalent ions are more than the concentration required to confer stability to BSNA adducts on DNA, it explains, at least in part, why habitual BN chewers are at high risk of stable adduct formation on their genetic material and consequent risk of mutagenesis/carcinogenesis (Chen et al., 1999; Liu et al., 2004). It has been shown that the risk of carcinogenesis progressively increased under continuing environment of BN exposure (Table 15.2). Possible biological consequence of adduct formation on DNA as well as damage inflicted upon the genetic material due to presence of such adducts could be many. At first, adducts on DNA may induce strand break, induce CA, SCE, UDS, etc. Secondly, damage to the genetic materials may cause alteration in pattern of gene expression. In particular, the changes in tumor suppressor genes *TP*53, *BRCA*1 and *BRCA*2 either by way of TP53 stabilization or mutation in critical domains are likely to diminish their tumor suppressor properties and favor carcinogenesis (Fig. 15.3). Thirdly, BN and their constituents, especially arecoline, has been shown to differentially dysregulate cell cycle control, mitochondrial membrane potential, GSH level and intracellular H_2O_2 production in the pathogenesis of OSF and oral cancer (Chang et al., 2001d). Reduction of GSH content by arecoline and BN extract and enhanced cytochrome P450 activity, which were observed in the liver of mice treated with BN, could cause increased oxidative metabolism of carcinogens and reduced detoxification. GSH depletion leads to increased oxidative stress that can cause DNA damage and trigger several response signals implicated in the carcinogenic process (Liu et al., 1996; Liu and Chi, 1999; Nair et al., 2004). Thus, BN and its constituents potentially interfere with cell signaling pathways. Little is understood about these aspects and more research is needed to unravel the influence of BN exposure on the complex cell signaling pathways. In spite of this, it is known that

BQ chewing contributes to the pathogenesis of cancer and OSF also by impairing T cell activation and by induction of PGE2, TNF-α and IL-6 production, which favors oral mucosal inflammation and growth of OMF and oral epithelial cells (Jeng et al., 2003). Similar end may also be achieved by activation of the MEK1/ERK/c-Fos pathway, which promotes keratinocyte inflammation, cell survival, and affects cell cycle progression (Chang et al. 2004). Alternatively, MMP-2, an enzyme belonging to matrix metalloproteinases (MMP) group of proteins that degrade extracellular matrix proteins and contribute to the tumor invasion and metastasis, was found to be elevated in most oral tumor patients with long term BQ usage while short term BQ usage increased the secretion of MMP-2 by oral epithelial cells and fibroblasts. This is suggestive of BQ consumption promoting oral tumor progression through the induction of MMP-2 secretion (Kato et al., 2005, Liu et al., 2005a). Elevation of MMP-9 was also observed following BQ chewing showing its role in the pathogenesis of oral mucosal lesions (Liu et al., 2005b). Due to this, levels of both MMP-2 and MMP-9 have been suggested as possible markers of human oral cancer (Patel et al., 2007). In all this, it has to be kept in mind that mere exposure to BN does not commit a cell or an organism to carcinogenesis. There are metabolic escape routes available to the exposed cell or organism by way of complete repair of damage and attainment of normalcy or necrotic or apoptotic programmed cell death (Fig. 15.6). Metabolic, cellular and other genetic factors, in complex and largely unclear ways, influence the path of carcinogenesis triggered by exposure to BN.

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