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REVIEW ARTICLE

Genetic instability and oral cancer

No-Hee Park*
School of Dentistry and Dental Research Institute
University of California, Los Angeles, CA 90095
E-mail : npark@dent.ucla.edu

Mo K. Kang
School of Dentistry and Dental Research Institute
University of California, Los Angeles, CA 90095
E-mail : npark@dent.ucla.edu

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Development of oral cancer proceeds through discrete molecular genetic changes that are acquired from the loss of genomic integrity after continued exposure to environmental risk factors. Of particular importance in oral cancer development are tobacco-related chemical carcinogens and human papillomavirus (HPV) infection. To understand the mechanisms by which these risk factors contribute to tumorigenesis, we developed an in vitro model of sequential, multistep oral carcinogenesis model of normal human oral keratinocytes (NHOK) by immortalizing these cells with cloned "high risk" HPV genome. HPV viral genome alone failed to give rise to a tumorigenic cell population, which required further exposure to chemical carcinogens. HPV-immortalized cells exhibited impaired cell cycle control and DNA repair activity upon exposure to DNA damaging agents, and accumulated elevated frequency of spontaneous and mutagen-induced mutation. Furthermore, expression of E6 and E7 oncoproteins of "high risk" HPV were found to be sufficient for the enhanced mutation frequency in NHOK. These findings suggest that viral infection in combination with existing chemical carcinogens may be the paramount causative agents for the induction of genetic instability and development of oral cancer.

Epidemiology of human oral cancer

Oral cancer imposes a significant health problem in the U.S. Its incidence accounts for 3-5% of all cancers, resulting in greater than 8,000 deaths (Gerson, 1990; White et al., 1995). The overall five-year survival rate of patients with oral malignancies is approximately 50% for Caucasian Americans and 31% for African Americans. From the 1940's through the early 1980's, the rate of oral cancer incidence has increased more than 50% in both men and women in the United States (Devesa et al., 1987). Besides the high mortality rates, oral cancer is frequently associated with oral and maxillofacial defects resulting from highly invasive surgical and radiation treatments. Consequently, oral cancer leads to devastating impacts to the patients' psychosocial, as well as physical, welfare.

Etiologic factors of oral cancer

Tobacco-Related Chemical Carcinogens. Numerous epidemiological studies have pointed the linkage between oral cancer development and extensive use of tobacco, either in the form of smokeless tobacco or cigarette smoking (Winn 1984; Preston and Correa, 1989; Hoffmann et al., 1991; Stich et al., 1992). Tobacco consumption is positively correlated with accumulation of DNA damage, and exposure to tobacco-related chemical carcinogens could provide direct damaging effects on the cellular DNA in the human oral cavity (Preston-Martin and Correa, 1989; De Stefani et al., 1990; Talamni et al., 1990; Stich et al., 1992; Phillips, 1996; Martin et al., 1996). DNA damaging agents found in tobacco include benzo(a)pyrene (B(a)P) and tobacco-specific N'-nitrosamines (TSNAs). Examples of TSNAs are N-nitrosonornicotine (NNN) and 4-[methylnitrosoamino]-1-[3-pyridyl]-1-butanone (NNK) (reviewed in Preston-Martin, 1991), and these chemicals exhibit carcinogenicity in animals (Huberman et al., 1976; Hoffmann et al., 1982; Preston-Martin and Correa, 1989). In fact, damaged genomic DNA has been detected as DNA-adducts in various tissues of cigarette smokers (Baan et al., 1988; Pulera et al., 1997). These findings strongly suggested a causal role of tobacco use in oral carcinogenesis.

However, continued intraoral placement of smokeless tobacco failed to evoke malignant conversion of oral mucosal cells of animals in vivo, indicating that tobacco use alone may not suffice development of oral cancer (Shklar et al., 1985; Park et al., 1986). Hence, other environmental factors including alcohol consumption, nutritional deficiencies, and DNA tumor viruses have also been implicated in oral carcinogenesis. Among these factors, human papillomavirus (HPV) gained much attention because (1) it is frequently found in oral cancer specimens, and (2) HPV is the utmostly important causative agent for human cervical cancer (reviewed in zur Hausen, 1996).

*Corresponding author

"High Risk" Human Papillomaviruses (HPV). Like genital cancer, HPV is also closely associated with benign and malignant oral lesions; HPV is detected in squamous cell papilloma, condylomas, focal epithelial hyperplasia and malignant oral lesions (Chang *et al*., 1990; Jalal *et al*., 1992; Syrjänen, 1992; Anderson *et al*., 1994; Ostwald *et al.,* 1994, Chiba *et al*., 1996; Paz *et al*., 1997; Wen *et al.,* 1997). Moreover, 46-78% of oral cancer biopsies contain HPV viral DNA (Woods *et al.,* 1993; Miller and White, 1996; Franceschi *et al*., 1996). Importantly, however, tissue specimens of normal histology occasionally contain HPV DNA (Syrjänen, 1992) and that only a small fraction of HPV-infected lesions rarely proceed to malignant transformation (zur Hausen, 1996b). Hence, these studies indicate that completion of tumorigenic conversion requires the presence of other risk factors than HPV infection alone, potentially tobacco-related chemical carcinogens.

Table 1. Mutation frequencies of pS189 shuttle vector plasmid in NHOK, HPV-immortalized HOK (HOK-16B, HOK-18A, and HOK-18C), and SCC-4 cells*.

Host Cells	Concentration of MNNG (mg/ml)	Plasmid Recovered # of Mutants/# of Total Colonies	Mutagenesis Frequency (%)
NHOK	0	8/16,785	0.047
NHOK	0.5	14/14,750	0.095
NHOK	1.5	27/15,048	0.179
HOK-16B	0	18/16,872	0.107†
HOK-16B	0.5	71/20,534	0.346††
HOK-16B	1.5	85/15,187	0.560†††
HOK-18A	0	40/22,538	0.178†
HOK-18A	0.5	121/21,080	0.574††
HOK-18A	1.5	146/16,631	0.878†††
HOK-18C	0	23/15,880	0.150†
HOK-18C	0.5	102/18,078	0.546††
HOK-18C	1.5	111/15,080	0.736†††
SCC-4	0	112/18,003	0.622†
SCC-4	0.5	134/18,930	0.708††
SCC-4	1.5	140/15,098	0.927†††

†Significantly higher than the mutation frequency in unexposed NHOK (Fisher's exact test, double tail).
††Significantly higher than the mutation frequency in NHOK exposed to MNNG (0.5 m g/ml) (Fisher's exact test, double tail).
†††Significantly higher than the mutation frequency in NHOK exposed to MNNG (1.5 m g/ml) (Fisher's exact test, double tail).
*published in Shin *et al*., 1996.

In vitro multistep oral carcinogenesis model

During carcinogenesis, normal cells undergo discrete, irreversible changes, including extension of normal life span, cellular immortalization, and tumorigenic transformation (Bishop, 1991; Shay and Wright, 1989; Gollahon and Shay, 1996; Guo *et al*., 1998). We have developed an *in vitro* model of sequential, multistep oral carcinogenesis model of normal human oral keratinocytes (NHOK) by immortalizing these cells with cloned "high risk" HPV genome. These immortalized HOK (1) contain integrated HPV DNA in cellular genome, (2) express viral oncogenes E6 and E7, and (3) contain extremely low level of wild-type (wt) p53 protein (Park *et al.,* 1991; Shin *et al.,* 1994). These cells were found non-tumorigenic in nude mice, but were transformed into tumorigenic cells after exposure to tobacco-related chemical carcinogens (Li *et al.,* 1992a and 1992b; Kim *et al*., 1993; Shin *et al*., 1994; Park *et al.,* 1995).

The sequential progression of NHOK to tumorigenic cells is illustrated in the organotypic "raft" culture, which allows the formation of keratinizing stratum corneum and differentiation of NHOK. However, HOK-16B (immortalized HOK cells with cloned HPV type 16 genome) showed abnormal differentiation properties, with crowding and a darkly stained basal layer, and HOK-16B-BaP (HOK-16B cells exposed to B(a)P for seven days) exhibited a disorganized culture pattern. Furthermore, HOK-16B-BaP-T (HOK-16B cells exposed to B(a)P for six months) and HOK-16B-BaP-T1 (derived from a tumor induced by HOK-16B-BaP-T cells in nude mice) were more crowded than the parental HOK-16B-BaP cultures. It is also notable that HPV-harboring cells failed to complete differentiation in "raft" culture.

Molecular basis of oral carcinogenesis

The detailed genetic alterations that elicit the phenotypic changes during our carcinogenesis model remain unclear, but overexpression of HPV E6/E7 genes were detected in the tumorigenic cells, indicating the possibility that these viral oncogenes play crucial roles in the tumorigenic conversion of NHOK (Chen *et al.,* 1997). E6 protein from "high risk" HPV interact with E6-associated protein (E6-AP), and the E6/E6-AP complex binds to and induces degradation of p53 (Huibregtse *et al*., 1993; Scheffner *et al*., 1993). In mammalian cells, wt p53 protein is involved in cell cycle arrest at G_1 upon exposure of cells to genotoxic stress, and allows repair of the damaged DNA before propagation of mutation via DNA replication (Livingstone *et al.,* 1992; Yin *et al.,* 1992).

Thus, wt p53 plays the pivotal role in the checkpoint mechanism against accumulation of mutations and consequent loss of genomic integrity. However, in HPV-immortalized cells treatment of cells with actinomycin D failed to evoke cell cycle arrest, while the normal counterpart demonstrated transient G_1 arrest. These data indicated that conversion of HPV-immortalized oral keratinocytes to tumorigenic cells may be due, in part, to the cells' inability to arrest the cell cycle when exposed to genotoxic agents, which would result in improper repair of damaged DNA.

Eukaryotic cells have multiple mechanisms for repairing damaged DNA, which include a nucleotide excision repair

(NER) system and base excision repair (BER) system, and the O^6 methylguanine DNA methyltransferase (MGMT) system. We have recently demonstrated that the induction of NER activity in HPV-immortalized HOK is significantly delayed compared to that in NHOK after exposure to UV-irradiation (Rey *et al*., 1999). The cells were irradiated with 2.5 J/m^2, and the DNAs were treated with T4 endonuclease V, which degrades DNA containing UV-induced cyclobutane pyrimidine dimers (CPDs). Within 8 h after UV-irradiation, less than 5% of either DNA strands was repaired in HOK-16B cells, while 60% of the transcribed and 20% of the non-transcribed DNA strands were repaired in NHOK. By 24 h after UV-irradiation, approximately 50% of the transcribed strand remained not repaired in HOK-16B cells when the repair of CPD was almost complete in NHOK. Numerous reports suggested the hypothesis that tumorigenesis progresses via a generalized increase in the rate of error during DNA replication, namely genetic instability (Nelson and Mason, 1972; Wheldon and Kirk, 1973; Loeb *et al*., 1974; Cairns, 1975). The original theory of genetic instability focused on the infidelity of DNA polymerases during replication, but the importance of DNA damage repair mechanisms for maintaining the stability of genome has subsequently been recognized (Ishwad *et al*., 1995). Genomic integrity of cells, in general, is maintained by the cell's ability to: (1) appraise the status of the genome at a given time point; (2) provide signals to proceed with or halt cell cycle progression; and (3) repair damaged DNA. Checkpoint genes coordinate cell cycle progression with cellular signals and allow the maintenance of genomic integrity. Inasmuch as our previous work suggest impaired cell cycle checkpoint and DNA damage repair mechanism in HPV-immortalized cells exposed to genotoxic stress, we have investigated the direct role of "high risk" HPV in oral carcinogenesis in the context of inducing genetic instability.

Table 2. Pattern of mutations of pS189 rescued from unexposed or MNNG-exposed NHOK, HOK-16B, HOK-18A, and SCC-4 cells*.

Type of		Mutation showing altered gel mobility	
Cells	Point mutation	Deletions	Complex mutations
NHOK			
S (n=8)	0	0	8 (100%)
M (n=25)	1 (4%)	0	24 (96%)
HOK-16B			
S (n=50)	1 (2%)	2 (4%)	47 (94%)
M (n=50)	8 (16%)	4 (8%)	38 (76%)
HOK-18A			
S (n=50)	1 (2%)	1 (2%)	48 (96%)
M (n=50)	5 (10%)	1 (2%)	44 (88%)
SCC-4			
S (n=50)	2 (4%)	2 (4%)	46 (92%)
M (n=50)	9 (18%)	8 (16%)	33 (66%)

S: spontaneous mutations
M: MNNG-induced mutations

To analyze the nature of spontaneous and MNNG-induced mutations in these various cell types, the plasmids were recovered from white and light-blue bacterial colonies and classified as point or complex mutations. The patterns of *EcoRI/BamHI* double digested fragments were compared with those of wt pS189 fragments. The fragment containing intact supF sequence is 850 base pairs in the absence of insertion or deletion. When mutant plasmids showed an identical digestion pattern with wt pS189, the mutants were classified as point mutation, while mutant plasmids showing different *EcoRI/BamHI* digestion pattern were classified as plasmids with altered gel mobility. Among altered gel mobility, plasmids showing an identical pattern except shorter supF fragment were classified as deletion, and plasmids which were not included in the above categories were classified as complex mutations. Mutant plasmids classified as point mutation and deletion were also confirmed by nucleotide sequencing as shown in Table 3.
* published in Shin *et al*., 1996.

"High Risk" HPV and genetic instability

The basal and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced mutation frequencies of NHOK and HPV-immortalized HOK were determined using shuttle vector plasmid pS189 (Shin *et al*., 1996). Successful use of this vector to detect mutation frequency has been well documented in a number of different experimental systems (Bredberg *et al.*, 1986; Seidman, 1986; Seidman *et al.*, 1987; Shillitoe *et al*., 1993). pS189 vector contains suppressor tRNA, *supF*, as a target for mutagens, and its activity is monitored in the *E. coli* strain MBM7070 that carries a *lacZ* amber mutation. In the presence of isopropylthio-β-D-galactoside (IPTG), 5-bromo-4-chloro-3-indolyl-13-D-galactoside (X-Gal) and ampicillin, *E. coil* MBM7070 forms blue colonies if *supF* gene contains no mutation. White colonies are formed, however, in the presence of mutation within the *supF* region (Shillitoe *et al.*, 1993). The spontaneous mutation frequency in the HPV-immortalized cells and cancer cell lines were approximately two and eight to twelve times higher than those in NHOK, respectively. Also, MNNG-induced mutation notably enhanced the mutation frequency of the plasmid in all tested cells; however, the frequency in the HPV-immortalized cells and cancer cells were three to five times and five to six times higher, respectively, than those in NHOK (Table 1). All eight mutant plasmids isolated from NHOK contained complex mutations (Table 2). Of the 25 mutant plasmids isolated from the MNNG-exposed NHOK, one plasmid contained a point mutation in the *supF* region and the remaining 24 plasmids showed complex mutations. Among 50 mutant plasmids isolated from the unexposed HOK-16B cells, 47 plasmids demonstrated complex mutations, 2 demonstrated deletions, and 1 showed a point mutation in *supF* region of the shuttle vector. When HOK-16B cells were exposed to MNNG, the percentage of mutant plasmids containing a point mutation of *supF* was significantly enhanced. Among 50 mutant

vectors, we observed 8 point mutations, 4 deletions, and 38 complex mutations. Of 50 plasmids mutated in the unexposed HOK-18A cells, 48, 1, and 1 vectors contained complex mutations, deletion, and point mutation, respectively, at the *supF* region, whereas the MNNG exposure significantly increased the number of plasmids with point mutations in the same cells. The mutational spectrum of the plasmids in the SCC-4 cells was similar to that in the HPV-immortalized cells, and the frequencies of point mutation and deletion of *supF* gene were notably enhanced when the SCC-4 cells were exposed to MNNG.

Table 3. Background and MNNG-induced mutation frequencies in the *supF* sequence of pS189 shuttle vector plasmids.Mutation Frequency (%)

Host Cells	MNNG	# of mutant colonies/# of total colonies	Mutation Frequency(%)
NHOK	-	13/25,406	0.051
NHOK	+	33/20,290	0.163
HOK/LXSN	-	12/18,878	0.064
HOK/LXSN	+	37/24,177	0.153
HOK/16E6	-	30/17,294	0.173[a]
HOK/16F6	+	105/17,764	0.591[b]
HOK/16E7	-	22/18,665	0.118[a]
HOK/16E7	+	90/21,846	0.412[b]
HOK/16E6/E7	-	33/18,660	0.177[a]
HOK/16F6/E7	+	123/18,769	0.655[b]
HOK/6bE6	-	18/19,504	0.092[c]
HOK/6bE6	+	35/15,368	0.228
HOK/6bE7	-	10/18,972	0.053
HOK/6bE7	+	31/18,843	0.165

[a] Significantly different from NHOK (-) group (Binomial comparison of two Poisson rates)
[b] Significantly different from NHOK (+) group (Binomial comparison).
[c] Not significantly different from NHOK(-) group (Binomial comparison).
Cells transfected with pS189 were exposed to MNNG (1.0 μ g/ml) for 2 hours and incubated in fresh medium for 24 hours.
The pS189 plasmids were recovered and intro.

E6 and E7 Viral Oncogenes and Genetic Instability

To understand the mechanism by which the mutation frequency is elevated in HPV-immortalized cells, we determined whether expression of E6 and/or E7 viral oncogenes is sufficient to elicit genetic instability in NHOK (Liu *et al*., 1997). Secondary NHOK cultures were infected with retrovirus expressing either E6 (16E6), E7 (16E7), or in combination of both (16E6/E7), and the basal and MNNG-induced mutation frequencies were determined using pS189 shuttle vector as described above. For comparison, parallel experiments were performed with E6 and E7 genes, denoted as 6bE6 and 6bE7, respectively, from type-6 ("low risk") HPV. The spontaneous mutation frequencies in 16E6, 16E7, and 16E6/E7 groups were significantly higher than in NHOK or control NHOK-LXSN, while no significant changes were detected in NHOK expressing 6bE6 and/or 6bE7 (Table 3). Likewise, MNNG-induced mutation frequencies were elevated by 16E6 and 16E7 oncogenes.

The mutation spectrum of *supF* region was determined by evaluating the electrophoretic mobility and nucleotide sequence, and classified as point mutation, deletion, or insertion. As shown in Table 4, spontaneous mutations in NHOK, NHOK-LXSN, NHOK-6bE6, and NHOK-6bE7 were equally distributed among different types of mutation, and MNNG treatment drastically induced the frequency of point mutation. However, NHOK expressing 16E6 and/or 16E7 demonstrated significant number of deletions and insertions in the presence or absence of MNNG-exposure. These data suggest that E6 and E7 oncogenes of "high risk" HPV are mutagenic in NHOK and further enhance the mutagenicity of carcinogen MNNG. From the above series of experiments, we conclude that "high risk" HPV induce genetic instability, which could be responsible for the greater susceptibility to tobacco-related chemical carcinogens in HPV-immortalized cells.

Concluding remarks

This review provides a broad discussion of environmental and cellular factors associated with oral carcinogenesis. Under continuous challenges imposed by environmental factors, normal human oral epithelial cells undergo constant cell division leading to regeneration of tissue, provided that the cells retain their ability to (1) limit their replicative life span through cellular senescence, (2) induce cell cycle arrest upon DNA damaged, and (3) repair the damaged DNA before resuming the cell cycle. "High risk" HPV infection, however, directly abrogates the innate checkpoint mechanisms against such environmental challenge, resulting in the accumulation and propagation of mutations. Hence, the viral infection in combination with existing chemical carcinogens may be the paramount causative agents for the development of oral cancer.

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