ORAL MICROBIOLOGY AND IMMUNOLOGY

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Loss of human β-defensin 1, 2, and 3 expression in oral squamous cell carcinoma

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Introduction: Human β-defensins (HBDs) are cationic, antimicrobial peptides produced by epithelial cells and involved in various aspects of the innate and acquired immune responses. They are expressed by oral tissues as constitutive and inducible genes. Recently, single nucleotide polymorphisms (SNPs) of β-defensins have been correlated with increased susceptibility to certain diseases. Studies have reported altered expression of β-defensins in cancers suggesting their involvement in carcinogenesis. The purpose of this study was to evaluate the regulation of HBD-1 (also published as DEFB1), HBD-2 (DEFB4) and HBD-3 (DEFB103A) (http://www.genenames.org/index.html) and HBD-1 SNPs in oral squamous cell carcinoma cell lines (OSCC) and healthy gingival keratinocytes.

Methods: β-defensin expression was quantitatively assessed using real-time polymerase chain reactions in OSCC and control cell lines after exposure to interleukin-1β, tumor necrosis factor-α, and interferon-γ. Control data were obtained in a previous study. DNA from 19 OSCC cell lines and 44 control subjects were extracted and the HBD-1 region spanning the 5′ untranslated region to the first intron was sequenced and analysed for SNP identification and distribution.

Results: HBD-1 and HBD-2 basal messenger RNA expression were significantly lower in OSCC. In addition, the ability to be induced was significantly reduced in OSCC for all three β-defensins. Four HBD-1 SNPs were differentially distributed between cancer and control populations. Genotype distribution at the HBD-1 locus also suggested loss of heterozygosity in OSCC.

Conclusions: The genetic variation observed in OSCC compared with that in control cell lines may account for differences in β-defensin expression. These results suggest a putative role for β-defensins in carcinogenesis and indicate that β-defensins may be useful markers of OSCC.

Key words: β-defensins; linkage disequilibrium; loss of heterozygosity; oral cancer; single nucleotide polymorphisms

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Activation of the innate immune response in the oral cavity includes expression of pluripotent molecules. Among these are human β-defensins, cationic broad-spectrum antimicrobial peptides active against gram-positive and gram-negative bacteria, fungi, and viruses (17, 24). In addition, β-defensins have been shown to have immunoenhancing, inflammation-modulating, and wound-healing capabilities (9, 41, 48). They are widely expressed throughout the body (17). In the oral cavity, β-defensin expression has been localized to mucosa, including gingiva and salivary glands (10) and is regulated by numerous stimuli including microorganisms (10, 13, 45) and proinflammatory cytokines (25). We previously described the heterogeneity and variability of basal and stimulated (induced) expression of β-defensins in primary keratinocyte cell lines (25). This heterogeneity suggests that different phenotypic profiles exist that may affect an individual's susceptibility or resistance to disease.

β-defensin expression is also regulated during inflammation *in vivo* (30) and is diminished in Crohn's disease, atopic dermatitis, inflamed airways due to allergy, and periodontal disease (5, 6, 21, 47). This β-defensin deficiency likely results in bacterial colonization which, in turn, stimulates inflammation (47).

Mechanisms leading to β-defensin deficiencies are not known; however, several studies suggest that genotypic polymorphisms could be responsible for such a phenomenon. β-defensin point mutations or single nucleotide polymorphism (SNPs) have been associated with multiple diseases including human immunodeficiency virus type 1 infection (8), chronic obstructive pulmonary disease (22), asthma (29), *Candida* carriage (26), and cystic fibrosis (46). A second source of genetic variance includes variability in gene copy number. Of the β-defensin genes, HBD-2, -3 and -4 have been shown to have from 2 to 12 copies of the gene (7, 20). Gene copy number, in turn, was found to correlate with HBD-2 messenger RNA (mRNA) expression (20).

The expression of β-defensins in cancer is controversial. For example, diminished HBD-1 expression has been reported for renal and prostate cancer (11, 49) and for basal cell carcinoma (16). In contrast, elevated HBD-1 expression has been reported for renal cell carcinomas (39) and in the serum of patients with lung cancer (along with upregulation of HBD-2) (3). HBD-3 expression has been shown to be increased in vulval squamous cell carcinoma (40). Data on β-defensin expression in oral squamous cell carcinoma (OSCC) are also conflicting. Low levels of HBD-2 expression in OSCC have been linked to poor differentiation (1). In contrast, other studies have reported increased HBD-2 expression in OSCC compared with normal epithelial cells (38). Because the levels of inflammation and associated cytokine production linked to cancer may vary, this could explain the conflicting results of β-defensin expression observed in previous studies. Preliminary data in our laboratory suggested diminished expression of HBD-1 in OSCC. Therefore, the purpose of this study was to: (i) evaluate the expression and regulation of HBD-1, -2, and -3 from a well-characterized collection of OSCC lines and compare them with previously characterized normal primary keratinocyte cell lines (25) and (ii) evaluate whether altered expression is associated with specific genetic polymorphisms at the HBD-1 locus.

Materials and methods

Human oral squamous cell carcinoma and normal primary gingival keratinocyte cell lines

A collection of 19 established human oral and oropharyngeal squamous cell carcinoma (OSCC) cell lines (cancer population) were obtained from several sources (American Type Culture Collection, Rockville, MD; Dr T. Carey, University of Michigan, Ann Arbor, MI; Dr P. Sacks, New York University, New York City, NY) and were grown in Dulbecco's modified Eagle's minimal essential medium with Ham's F12 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The control population comprised 44 samples from different subjects obtained using (i) buccal swabs collected from healthy subjects using Catch-All™ (Epicentre, Madison, WI) and (ii) normal primary oral keratinocyte cell lines isolated from healthy subjects undergoing tissue resective surgery for dental restorative purposes. Gingival keratinocytes were grown and cultured as previously described (25). In addition, a normal primary tonsillar keratinocyte cell line HTE1163 (Drs A. Klingelhutz and J. Lee, University of Iowa) was grown in keratinocyte serum free medium with 0.2 ng/ml epidermal growth factor and 30 μg/ml bovine pituitary extract (GIBCO). All cultures were grown in a humidified incubator at 37°C under 7.5% CO_2 in air. All tissue samples were obtained in compliance with a protocol approved by the University of Iowa Institutional Review Board for the Use of Human Subjects in Research.

Cell culture stimulation

To assess the inducibility of the β-defensins in OSCC, cells were seeded into 12-well plates (Corning Inc., Corning, NY) at a density of 1×10^5 cells per well and grown overnight. Cells were then exposed to the following mediators, alone or in combination: interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) (100 ng/ml) and to low-dose (200 ng/ml) or high-dose (10 μg/ml) *Escherichia coli* lipopolysaccharide (LPS) for 24 h in keratinocyte growth medium without serum (Clonetics, Biowhittaker Inc., Walkersville, MD). After 24 h, cells were collected and RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH). This protocol has been previously published (25) and the data from that study was used for comparison in the present study.

Reverse transcription-polymerase chain reactions (RT-PCR)

Total RNA was reverse transcribed in a final volume of 20 μl using the superscript system according to the manufacturer's instructions (Superscript™ II RNase H reverse transcription system; Invitrogen, Carlsbad, CA). Briefly, 2 μg RNA, 200 U SuperScript™ II, 4 μl 5 × first strand buffer, 500 ng Oligo(dt)12 (Integrated DNA Technology, Coralville, IA), 0.01 M dithiothreitol (GIBCO), and 1 μl 10 mM dNTP mix (Roche, Indianapolis, IN) were incubated at 42°C for 52 min. The resultant complementary DNA (cDNA) from the RT-PCR served as a template for real-time PCR amplifications.

Real-time PCR

Real-time PCR analyses of HBD-1, -2, and -3 were performed with a fluorescence ABI Prism™ model 7700 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Probes and primers for HBD-1, -2, and -3 were designed and developed previously (25). To normalize the mRNA detection level, the housekeeper gene for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was included in the analysis. Each probe was synthesized and labeled at the 5′ end with a reporter dye and at the 3′ end with the quencher dye. Reactions with a final volume of 25 μl containing 250 nM HBD-1, HBD-2, or HBD-3 probe, 500 nM of each HBD-1, HBD-2, HBD-3 primer, 125 nM of GAPDH probe (TaqMan®; Applied Biosystems), 250 nM of each GADPH primer (TaqMan®; Applied Biosystems), 1 μl RT reaction and 12.5 μl of the 2 × TaqMan® Universal master mix were performed in triplicate. Conditions for thermal cycling were 50°C for 2 min followed by 95°C for 10 min and 40 cycles of 15 s at 95°C and 60°C for 1 min.

Analysis of PCR products

Quantitative analysis was performed using a comparative Ct method comparing the threshold cycle (Ct, cycle number at which product was detected) of the gene of interest with the Ct generated by a reference sample (non-stimulated keratinocyte cultures). For each condition tested, gene expression was first normalized to GAPDH expression by subtraction of the GAPDH Ct value from the value obtained for the gene studied (ΔCt values). The ΔΔCt was then calculated as the difference between the ΔCt values from stimulated and non-stimulated keratinocytes. Relative differences in expression between stimulated and non-stimulated conditions within subjects represented the induction factor

and were calculated using the equation $2^{-\Delta\Delta Ct}$ (User bulletin#2; Applied Biosystems). When basal levels of expression were compared among subjects and not with a control or standard, the ΔCt was solely taken into account and an index was calculated using the formula described as: 1/ΔCt because a lower ΔCt represents more cDNA (25). Induction frequencies were calculated as a percentage of cell lines significantly induced within our population.

DNA isolation

DNA was extracted from buccal swabs and purified using the QuickExtract™ (Epicentre) DNA extraction kit according to the manufacturer's instructions. DNA from OSCC and primary gingival keratinocyte cell lines were extracted using the QIAamp DNA (Qiagen Inc., Valencia, CA) extraction kit. DNA concentrations were determined with the Nanodrop spectrophotometer (Nanodrop technologies, Wilmington, DE) based on the optical density reading at 260 nm (OD_{260}) reading. The DNA was stored at −20°C.

PCR amplification of genomic DNA

The HBD-1 regions spanning the 5′ untranslated region, exon 1, and the beginning of the intron were amplified using the following primer sets described by Jurevic et al. (27): 5′-AAGT TCACCCTTGACTGTGGCACC-3′ and 5′-GAGACTCACATCAGCCCCATTGTCC-3′ for HBD-1; 305 base pairs (bp). Each reaction contained 50 ng DNA, 1 × PCR buffer (Bioline, Randolph, MA), 1.5 mM $MgCl_2$, 200 μM each dNTP (Roche), 0.5 U *Taq* Polymerase (Bioline), 500 nM forward primer, and 500 nM reverse primer in a final reaction volume of 100 μl. An initial denaturation step (94°C, 3 min) was followed by 35 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s), extension (72°C, 30 s), and elongation (72°C, 5 min).

Gel electrophoresis

Amplified products were run on a 1.5% agarose gel in 1 × TAE buffer (0.8 mM Tris–acetate, 0.04 mM disodium ethylenediamine tetraacetate–$2H_2O$, pH 8.5). Gels were stained with ethidium bromide and visualized using ultraviolet illumination (Foto UV21; Fotodyne, Hartland, WI). A 100-bp ladder (GIBCO) was used to assess the molecular weight of the PCR products. Bands corresponding to the expected sizes were excised, extracted, and purified from the gel using a QIAquick Gel extraction kit (Qiagen Inc.). DNA concentrations were determined with the Nanodrop spectrophotometer (Nanodrop technologies) based on the OD_{260} reading.

Sequencing

Bidirectional sequencing of the amplified regions of HBD-1 was performed with the primers used for the initial amplifications. Sequencing was carried out using an ABI 3700 autosequencing system (Perkin-Elmer/Applied Biosystems, Foster City, CA) using the PCR cycle sequencing protocol and fluorescent dye terminator dideoxynucleotides (Perkin-Elmer/Applied Biosystems). SNPs were identified based upon the analysis of the chromatographs obtained using the FINCHTV software (http://www.geospiza.com/Products/finchtv.shtml). DNA sequence analyses and transcription factor binding sites were performed using the ACCELRYS WISCONSIN SOFTWARE PACKAGE.

Linkage studies

Linkage disequilibrium was assessed using the Index of Association as calculated by the MULTILOCUS 1.3 software package (http://www.agapow.net/software/multilocus/) (2). Significance was determined by comparing the observed results with multiple randomized datasets, simulating random mixing between loci (linkage equilibrium).

Statistical analysis

β-defensin mRNA expression was compared using the non-parametric Mann–Whitney test (comparison between control and cancer population) or Wilcoxon signed rank test when values were paired (comparison of gene expression within population). To determine the associations among defensin mRNA expression level, a Spearman rank correlation coefficient, rs, was used. Stimulation by inducers was compared to non-stimulated conditions using one-way analysis of variance. The significance was tested using Tukey's multiple comparison method with a significance value of $P < 0.05$. Allelic and genotypic frequencies were calculated for each polymorphic nucleotide site and Fisher's exact test was used to test the significance of the distribution between the control and the cancer populations. Hardy–Weinberg equilibrium was tested at each polymorphic site for each population. Because of small expected values, Fisher exact 3 × 2 contingency tables were used instead of chi-squared analyses to compare observed and expected values ($P < 0.05$). Loss of heterozygosity was evaluated by GENEPOP 3.3 software using the Markov chain method at http://genepop.curtin.edu.au.

Results

OSCC cell lines express low basal levels of β-defensins

Basal levels of expression for HBD-1 in OSCC were heterogeneous (Fig. 1A) whereas basal expression of HBD-2 and HBD-3 was consistent among the OSCC lines (Fig. 1B,C). Average basal expression levels of HBD-2 and HBD-3 were significantly lower than that of HBD-1 ($P = 0.001$ and $P = 0.0067$, respectively). This finding is similar to our previously published results for the control population (25). Interestingly, expression levels of the three defensin genes were highly correlated in cancer cell lines (HBD-1 and HBD-2, rs = 0.8132, $P = 0.0001$; HBD-1 and HBD-3, rs = 0.7882, $P = 0.002$ and HBD-2 and HBD-3, rs = 0.8587, $P < 0.0001$). In contrast, correlation of the β-defensins in the control population showed only moderate association for HBD-1 and HBD-3 (rs = 0.58, $P = 0.01$) (25). The mean basal expression of HBD-1 and HBD-2 was 2.03-fold and 1.32-fold greater, respectively, in the control population vs. cancer cell lines ($P = 0.0002$ and $P = 0.0062$, respectively) (Fig. 1A). HBD-3 expression in the control and cancer populations did not differ significantly ($P = 0.2455$).

β-defensin induction in OSCC is diminished

We previously demonstrated that β-defensins were regulated by IL-1β, TNF-α, and IFN-γ in normal cell lines and that the intensity of the response was both mediator-specific and defensin-specific and correlated with basal levels of expression (25). Overall β-defensin basal expression was reduced in OSCC compared with normal cell lines so we hypothesized that this would also affect their ability to be induced by various inflammatory mediators.

Overall, induced expression of the β-defensins was significantly decreased in OSCC cell lines when compared with the normal cell lines (Table 1 and Fig. 2). Upon cytokine stimulation, HBD-1 mRNA expression in the cancer cell lines was not significantly different from the

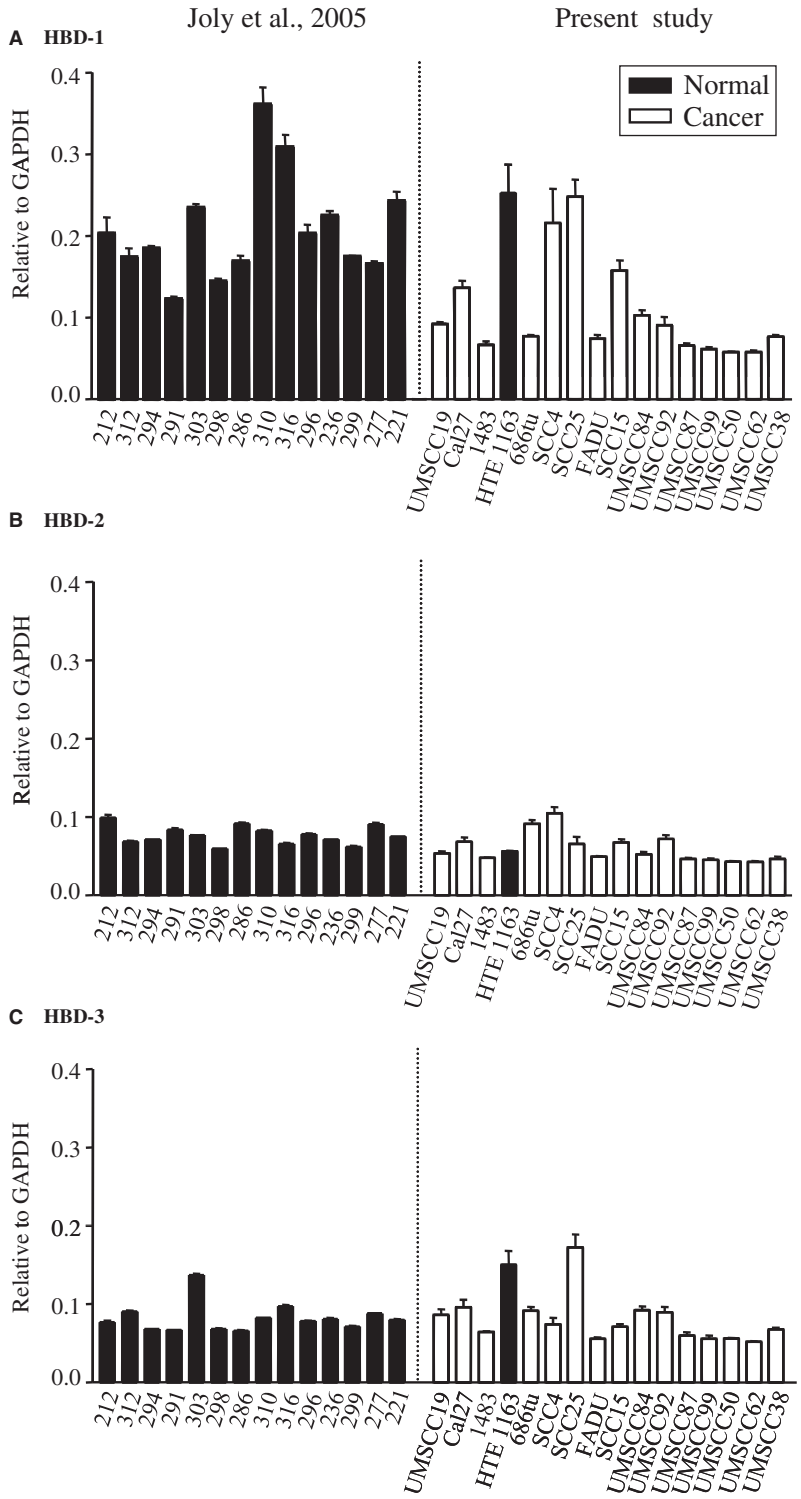

Fig. 1. Basal messenger RNA expressions level of (A) human β-defensin-1 (HBD-1), (B) HBD-2, and (C) HBD-3 for 15 normal cell lines (black bars) and 16 oral squamous cell carcinoma (OSCC) cell lines (white bars) as assessed by real-time polymerase chain reaction. Basal level of expression for each cell line (mean ± SEM representing duplicate wells) is presented as a ratio of defensin expression relative to the expression of the housekeeper gene, *GAPDH*. The normal cell lines, except HTE1163, were obtained and evaluated as a part of a previously published study (25).

non-stimulated control for each of the cell lines tested (Table 1 and Fig. 2A). Similar to the normal cell lines, IL-1β and TNF-α induced HBD-2 in most cancer cell lines; however, the magnitude of the response was significantly decreased compared with the normal cell lines (Table 1 and Fig. 2B) ($P < 0.0001$ and $P = 0.017$, respectively). In addition, only 53% and 67% of the OSCC cell lines were induced by IL-1β and TNF-α, respectively (Table 1). Similarly, HBD-3 induction frequency and levels of induction were significantly reduced in the OSCC cell lines compared with the normal cell lines when stimulated with IFN-γ ($P = 0.0004$) (Table 1). HBD-3 inducibility by IFN-γ averaged 41.2 for the cancer cell lines compared with 147 for the normal cell lines (Table 1). When cytokines were combined, synergism was evident; however, the magnitude of the synergistic response in the cancer cell lines was much less than that seen for the normal cells (Fig. 2). *E. coli* LPS did not stimulate the β-defensins in either the cancer or control cell lines (data not shown).

Overall, basal expression of the β-defensins did not correlate with the induction potential in the cancer cell lines. This contrasts with the strong correlations seen with IL1-β and IFN-γ inducibility relative to basal expression in the control cell lines (25).

Polymorphisms in HBD-1 show strong association with cancer

To assess whether the twofold reduction in basal expression and altered regulation of HBD-1 in OSCC cell lines was the result of specific mutations in the regulatory sequences, we examined a portion of the HBD-1 gene for SNPs in OSCC and normal cell lines. Mutations of HBD-2 and HBD-3 were not included in this study because they have multiple copies of the genes, which produces inaccuracies in the SNP analysis.

A 302-bp fragment was amplified containing partial promoter sequence and the first exon of HBD-1 using primers previously published (27). Four SNPs were identified by sequencing (−52, −44, −20, and 80) (Table 2). These SNPs corresponded to SNPs previously identified in the HBD-1 locus (27). The SNP frequencies in the control population (Table 2) matched those presented by Jurevic et al. (27). Genotypic and allelic frequencies computed for the different SNPs identified a strong association between SNPs and health status at each SNP site. Increased frequency of −44G and 80A was significantly associated with the cancer population [$P = 0.002$, odds ratios (OR) equal to 3.7] (Table 2). The GG and AA homozygotes for −44 and 80 SNPs respectively, had an even stronger correlation with the

Table 1. Comparison of human *β*-defensin-1, -2, and -3 expression following IL-1*β*, IFN-*γ*, or TNF-α stimulation in cancer versus normal control cell lines

	IL-1*β*				IFN-*γ*				TNF-α			
	Induction frequency (%)[1]		Induction factor[2]		Induction frequency (%)[1]		Induction factor[2]		Induction frequency (%)[1]		Induction factor[2]	
n[3]	C15	N15	C15	N15	C15	N15	C15	N15	C15	N7	C15	N7
HBD-1	0	20	NA	4	0	87	NA	5	0	14	NA	7
HBD-2	53	100	85	514	13	50	27[4]	8	67	86	30	216
HBD-3	33	67	6.5	7	33	93	41	147	47	71	27	9

[1]Percentage of cell lines that responded to cytokine challenge by upregulation of HBD-1, -2, or -3 gene expression. C represents the cancer cell lines analysed in this study and N represents the data obtained for normal control cell lines (25).
[2]Average amount of induction in cell lines presenting significant upregulation compared with non-stimulated conditions.
[3]*n* represents the number of cell lines analysed.
[4]Average of two cell lines with induction factors of 4.75 and 48.63.
HBD-1, human *β*-defensin-1; IFN-*γ*, interferon-*γ*; IL-1*β*, interleukin-1*β*; NA, not applicable; TNF-α, tumor necrosis factor-α.

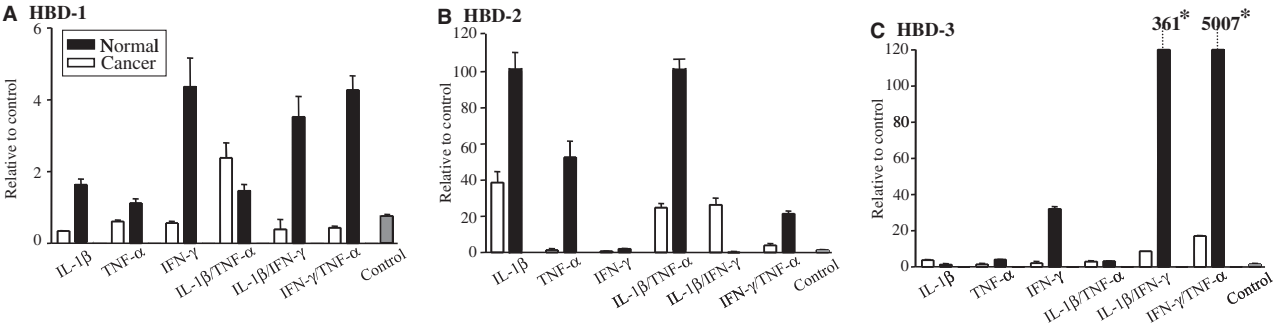

Fig. 2. Messenger RNA expression and regulation of a representative normal cell line (#310; black bars) and cancer cell line (#1483; white bars) compared to control (gray bars) for (A) human *β*-defensin-1 (HBD-1), (B) HBD-2, and (C) HBD-3. Induction factors obtained for the cell lines #310 and #1483 were representative of the responses seen with the other cell lines in their respective groups to the individual cytokines (Table 1). Stimulation was performed with various cytokines (alone or in combination): IFN-*γ*, interferon-*γ*; IL-1*β*, interleukin-1*β*; TNF-α, tumor necrosis factor-α. Control (white bar) = average value of $2^{-\Delta\Delta Ct}$ (see Materials and methods) obtained for non-stimulated conditions. Asterisk represents the induction factor.

Table 2. Allelic and genotypic frequencies of human *β*-defensin-1 single nucleotide polymorphisms

SNP[1]	Control (n = 44)	Cancer (n = 18)	P-values
−52 (G/A)			
G	0.69 (61/88)	0.86 (31/36)	0.07
A	0.31 (27/88)	0.14 (5/36)	
G/G	0.43 (19/44)	0.83 (15/18)	0.005
G/A	0.53 (23/44)	0.05 (1/18)	0.0005
A/A	0.04 (2/44)	0.12 (2/18)	0.57
−44 (C/G)			
C	0.78 (69/88)	0.50 (18/36)	0.002
G	0.22 (19/88)	0.50 (18/36)	
C/C	0.61 (27/44)	0.44 (8/18)	0.55
C/G	0.34 (15/44)	0.12 (2/18)	0.11
G/G	0.05 (2/44)	0.44 (8/18)	0.0002
−20 (G/A)			
A	0.51 (45/88)	0.42 (15/36)	0.6
G	0.49 (43/88)	0.58 (21/36)	
G/G	0.16 (7/44)	0.55 (10/18)	0.005
G/A	0.66 (29/44)	0.05 (1/18)	0.0001
A/A	0.18 (8/44)	0.39 (7/18)	0.038
80 (T/A)			
T	0.78 (69/88)	0.50 (18/36)	0.002
A	0.22 (19/88)	0.50 (18/36)	
T/T	0.61 (27/44)	0.44 (8/18)	0.55
T/A	0.34 (15/44)	0.12 (2/18)	0.11
A/A	0.05 (2/44)	0.44 (8/18)	0.0002

[1]Single nucleotide polymorphism (SNP) locations are presented as a negative number subtracted from the first base of the first exon's start codon.

cancer population (P = 0.0002, OR = 17) (Table 2). Allelic frequencies at the −52 and −20 SNPs were not significantly different between the cancer and control populations. However, the presence of heterozygotes was strongly associated with the control populations (OR = 19 for −52 SNP; OR = 33 for −20 SNP) (Table 2). In contrast, homozygotes at −52 or −20 sites were significantly associated with the cancer population (OR = 7) except for −52AA (Table 2). The presence of homozygotes for all four SNPs was highly correlated with cancer (P = 0.00001) with an OR of 24. Haplotypes were also compiled for the four HBD-1 SNPs. GGGA was significantly more represented in the cancer population (P = 0.0002, OR = 6.3) with a frequency of 0.334 compared to 0.07 in the control population (data not shown).

HBD-1 SNP frequencies in cancer deviate from Hardy–Weinberg law

While Hardy–Weinberg equilibrium was confirmed for the four HBD-1 SNPs in the control population, three SNPs (−44, −20, and 80) were not in a state of equilibrium in the cancer population (P = 0.046, P = 0.016, and P = 0.046, respectively). Furthermore, loss of heterozygosity was observed at all HBD-1 loci in the cancer population (P < 0.00001). No deficit of heterozygosity was found in the control population. Of the cancer cell lines, 89% presented homozygous profiles when all four HBD-1 SNPs were considered together, compared with 27% for the control cell lines. These results indicate a low level of heterozygosity for HBD-1 in cancer cells.

Evidence of increased linkage disequilibrium in the cancer population

Linkage disequilibrium (LD) was assessed for the two populations for SNPs observed at the HBD-1 locus. Results are presented in Fig. 3. While LDs were shown for both the cancer and the control samples, the pairs of loci in LD in the two groups differed significantly. A notable increase in LD was observed among HBD-1 SNPs in cancer cell lines. Out of the six HBD-1 pairwise comparisons, two pairs of loci

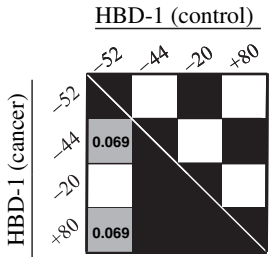

Fig. 3. Levels of significance for non-random association between loci (linkage disequilibrium). Pairwise linkage disequilibrium (LD) is plotted for four HBD-1 single nucleotide polymorphisms (SNPs), identified by their position relative to the START codon. Tests are based on randomization procedures (10^5 runs) that simulate random mixing between SNPs (null hypothesis). LDs for the normal and cancer populations are represented in the upper-right and the lower-left sections, respectively. Gray-scale reflects the significance of the *P*-value: dark squares indicate strong LD between pairs of SNPs, $P < 0.001$; light gray squares indicate marginal LD, $0.1 > P \geq 0.05$; white squares indicate no LD, $P \geq 0.1$. Exact *P*-values are indicated only for the light gray squares ($0.1 > P \geq 0.05$).

demonstrated LD in control cell lines. In cancer cell lines, five of the six pairwise comparisons indicated LD ($P < 0.05$) or near disequilibrium ($P = 0.069$). The unique pair demonstrating LD in the control but not the cancer group was −20/−52. Despite the presence of increased LD in the cancer sample, one SNP pair, −20/−52, demonstrated LD in the control but not the cancer group. These results further emphasize the notable differences observed between the two populations.

Discussion

β-defensins are believed to be involved in the maintenance of homeostasis in the oral cavity. Our results showed that the expression and regulation of HBD-1,- 2, and -3 is altered in oral cancer. Compared with normal cell lines, we demonstrated that basal mRNA expression was significantly reduced in OSCC for two of the three β-defensins studied and that the amount produced in response to stimulation by mediators was minimal for all three β-defensins in OSCC cell lines.

Decreased expression of HBD-2 in oral cancer cells has also been reported *in vivo* (1). In contrast, others have shown elevated levels of HBD-2 in OSCC (38). Our results suggest that this apparent contradiction may be explained by levels of inflammation in biopsy sections. Despite a decrease in frequency and amplitude of β-defensin induction in OSCC, upon stimulation with IL-1β, TNF-α, and IFN-γ, significant increases in β-defensin mRNA expression were still seen in some OSCC cell lines relative to control. This suggests that inflammation, which is not uncommon in carcinoma (4), could restore β-defensin expression levels to those seen as healthy basal levels (or more) in some cases. Because genetic polymorphism at transcription factor sites can result in decreased expression, we hypothesized that the diminished expression seen for the OSCC cell lines could be the result of polymorphisms in the promoter region.

Interestingly, we showed genetic divergence of control and cancer cell lines at the HBD-1 gene locus, which was suggestive of an association between genetic polymorphism and HBD-1 expression and regulation. A survey of the sequences encompassing the HBD-1 SNPs analysed in this study, did not show homology between SNP sites and putative transcription-factor-binding sites that could explain the decreased induction potential. However, linkage studies demonstrated a strong association between polymorphisms. Therefore it is possible that SNPs associated with cancer may be linked to other polymorphisms affecting transcription-factor-binding sites in neighboring sequences that were not analysed in this study.

The LD analysis also demonstrated significant differences between cancer and control cell lines. These differences could result from the presence of a deficit in heterozygotes, as indicated by the departure from Hardy–Weinberg equilibrium at the HBD-1 locus in our cancer population. Allelic imbalance and twofold reduced expression of HBD-1 in cancer strongly suggest a loss of heterozygosity at the HBD-1 locus and could be the consequence of chromosomal deletion around this gene cluster.

Preliminary data in our laboratory also demonstrate an allelic imbalance of HBD-2 in cancer. Because HBD-2 and HBD-3 are duplicated, a traditional genetic analysis of SNP is obsolete without copy number analysis. Analysis of HBD-2 and HBD-3 copy number will be included in future studies. A large deletion comprising the β-defensin gene cluster could result in a reduction in the number of gene copies and could in turn explain the decrease in inducibility observed for HBD-2 and HBD-3.

Departure from Hardy–Weinberg equilibrium is in agreement with the possible presence of hemizygotes at this locus. In this instance, the linkage disequilibrium study would still be valid because the test used is independent of the ploidy level.

Loss of heterozygosity in the chromosome 8p21–23 region is associated with many types of cancer (14, 31, 34, 35). Specifically, large deletions in the 8p21–22 and 23 regions are common in oral or head and neck squamous cell carcinoma and suggest that this region harbors tumor suppressor genes (12, 23, 35, 37, 44). However, unlike other types of cancer, few specific genes have been associated with head and neck cancer. One such gene, *LZTS1*, presents features similar to those presented here for HBD-1, with absent or reduced mRNA expression in primary OSCC associated with loss of heterozygosity (35). Our results support a recent report that presents HBD-1 as a potential tumor suppressor in renal cell carcinoma based on the fact that overexpression of HBD-1 induced cancer cell apoptosis (43) and that HBD-1 was down-regulated in renal cell carcinomas and malignant prostate samples (11). However, in contrast to our study, HBD-1 SNP frequencies were normal for the prostate cancer population and there was no evidence of loss of heterozygosity (11). This suggests that mechanisms leading to decreased HBD-1 expression could differ for different types of cancer.

Loss of heterozygosity at 8p assessed by HBD-1 SNP analysis could serve as an initial screening for cancer in oral premalignant lesions such as leukoplakia. Recently Zhou et al. (50) concluded that allelic imbalance for chromosomal region 8p was observed in 66.7% of oral premalignancies. Data suggest that genomic rearrangements occur at an early phase of tumor development and are essential events for cancer progression (42).

Our study and others present strong evidence of an association between β-defensin and oral cancer. However, the specific role of the β-defensins in carcinogenesis is unknown. Low levels of HBD-1 could decrease apoptosis (43), or drastically alter cell differentiation because HBD-1 stimulates keratinocyte differentiation (15). Alternatively, HBD-mediated activation of innate and acquired immunity might serve as an anti-cancer agent. For example, vaccination with a lymphoid leukemia cell line expressing the mouse β-defensin 2 gene (*MBD2*) resulted in protective immunity leading to an 80% rate of complete leukemia rejection when challenged with a parental leukemia line. This was in contrast to a 100% death rate in the group that did not receive the vaccination (32). Finally, as shown for other cationic peptides, such as cecropins and mangainins, β-defensins may have

cancer-selective cell membrane lytic effects or could specifically increase apoptosis in cancer cells through mitochondrial membrane disruption (36). Downregulation of these genes would therefore contribute to cancer survival and propagation.

It has also been proposed that *β*-defensin deficiency contributes to microbial invasion and infection (47). It is generally accepted that viruses and bacteria such as *Helicobacter pylori* contribute to carcinogenesis (20, 28). *β*-defensins are known to possess potent activity against *Candida* species and *H. pylori* (18, 24) both of which are associated with cancer or precancerous lesions (33). Hence, decreased *β*-defensin production could favor microbial colonization of tumor sites and contribute to cancer progression.

Whether *β*-defensins, specifically genetic polymorphisms, directly contribute to cancer cell differentiation and cancer progression, or whether their altered expression and regulation are a cause or result of malignant transformation, it is crucial to understand their role in oral cancer. Decreased levels of *β*-defensins affect (i) antimicrobial activity, (ii) wound healing, and (iii) immunoregulatory functions of both innate and adaptive immune responses, all of which may play a role in the pathogenesis of oral cancer.

Future genetic analysis of the *β*-defensin gene cluster with complete characterization of SNPs and gene copy number will be important in determining if these genetic markers may serve as diagnostic tools in screening individuals for risk of OSCC. Ultimately, restoration or augmentation of *β*-defensin production may have therapeutic application in the treatment of oral squamous cell carcinoma.

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