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Clin Cancer Res 2003;9:1057-1062. Published online March 1, 2003.

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ABSTRACT

Purpose: **Genomic instability plays a major role in the genesis and progression of tumors, and in the evolution of tumor heterogeneity. To determine the role of genomic instability in the genesis and progression of oral cancer, we assessed the extent of genomic alterations in oral squamous cell carcinomas (OSCCs).**

Experimental Design: **We used the recently developed inter-(simple sequence repeat) PCR technique to quantitate genomic instability using matched tumor and normal OSCC samples ($n = 25$). The inter-repeat region bands of similar molecular size observed to be altered in more than one case were sequenced and analyzed to identify probable OSSC-associated specific genetic lesions.**

Results: **Of the four base-anchored, dinucleotide repeat-based primers used for the study, the most informative profile in OSCCs was generated by the $(CA)_8RG$ primer. Measurement of genomic instability index using the $(CA)_8RG$ primer revealed a high incidence of genomic instability in OSCCs. No significant correlation between the extent of alterations and stage or location of the tumor was observed. Sequencing analysis of the altered bands revealed gains/losses in several chromosomal regions. Of the matched tumor and corresponding normal tissue DNA studied, hitherto unreported losses were seen in 11p15 and 17q25 chromosomal regions. Sequencing of some of the tumor-specific altered regions indicated that they code for regions of *UDP-GalNAc* and *hRAD 17* genes, which were lost (deleted) in oral cancer.**

Conclusions: **Our results indicate that the extent of genomic instability in OSCC is not correlated to the tumor stage or location. For the first time, we have shown that chromosomal alterations detected by inter-(simple sequence repeat) PCR could be correlated to genes associated with cancer development.**

INTRODUCTION

OSCC[3] is one of the most prevalent forms of cancer in the regions of world where tobacco chewing in combination with alcohol consumption is high. Many molecular aberrations occur in the genome of OSCCs such as mutations in tumor suppressor genes *p53*, *p16*, and *fhit*, and deletion in *p14 ARF*; and in addition a high rate of mutations in *Ha-ras* oncogene is also reported. Most of these mutations were characteristic of *N*-nitrosamine carcinogen damage in the DNA of OSCC patients (1–4).

Progression of tumors has been attributed to the cumulative accumulation of multiple alterations throughout the genome, which is manifested by genomic instability. A mutator phenotype has been proposed, which destabilizes the genome, and drives rapid progression of the tumor and continuous evolution of microheterogeneities within the tumor (5).

Measurement of genomic instability has been made using techniques like flow cytometry, fluorescent *in situ* hybridization, CGH, and allelotyping, which, although informative, are cumbersome to perform and, hence, impractical in the assessment of clinical cases (6).

In this study, we have attempted to assess genomic instability and possibly detect tumor-specific genomic alterations in OSCCs. We have used a rapid technique developed recently, ISSR-PCR, for measurement of genomic instability (6–12). Here, PCR primers homologous to dinucleotide repeats, anchored at the 3′ end by two nonrepetitive nucleotides were used to evaluate genomic instability. This was done by observing the appearance (gains) or disappearance (losses) of amplified DNA bands from tumors and matched normal tissues. ISSR-PCR is well suited to detect common genetic events observed in sporadic cancers like aneuploidy, wherein entire chromosomes are

Received 5/20/02; revised 10/28/02; accepted 10/28/02.
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[1] Supported by grants from the Department of Science and Technology, Government of India. M. V. is a research fellow of the Council of Scientific and Industrial Research, India. G. S. was an Indian National Science Academy Senior Scientist during this study.
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[3] The abbreviations used are: OSCC, oral squamous cell carcinoma; ISSR-PCR, inter-(simple sequence repeat) PCR; LOH, loss of heterozygosity; MIN, microsatellite instability; CIN, chromosomal instability; CGH, comparative genomic hybridization.

gained or lost, as well as intrachromosomal instabilities characterized by insertions, deletions, amplifications, and translocations. Furthermore, this technique is sensitive enough to detect small alterations not detectable by CGH or flow cytometry (6).

MATERIALS AND METHODS

Tumor Samples and Isolation of DNA. Consecutive biopsy or resection samples were collected along with normal tissue from adjacent region in PBS, and transported on ice and DNA isolated immediately as described (6). Briefly, 100 mg of tissue was cleared of debris, cut into small pieces of 2–3 mm, and digested overnight with 1 μg/ml proteinase K (Sigma) in proteinase K buffer (10 mM Tris-Cl, 5 mM EDTA, and 0.5% SDS) at 60°C. The samples were treated with 0.5 μg/ml RNase A (Sigma) for 30 min, and extracted twice with phenol and finally with chloroform:isoamyl alcohol. DNA was precipitated with 95% ethanol containing 0.1 M sodium acetate. The DNA was vacuum dried, reconstituted in Milli-Q H_2O, and quantitated spectrophotometrically.

ISSR-PCR. Fifty ng of the DNA sample was amplified in a 10 μl reaction containing 1× PCR buffer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 2.5 μM of one of the base-anchored dinucleotide repeat primers (Microsynth GmBH, Balgach, Switzerland), 0.5 units of TaqDNA polymerase (Perkin-Elmer), and 5 μCi of [α-P^{32}]dCTP.

The cycling conditions were 3 min initial denaturation at 94°C, 30 cycles of 30 s at 94°C, 45 s at 50°C, and 2 min at 72°C followed by final extension for 7 min at 72°C on a Perkin-Elmer GeneAmp 2400 thermocycler.

The PCR product (2.5 μl) was mixed with 0.5 μl 6× loading buffer (30% glycerol, 1.25 mM EDTA, 0.025% xylene cyanol, and 0.025% bromphenol blue). The products of tumor and the corresponding normal DNA samples were loaded onto adjacent lanes on a 0.4-mm thick 8% nondenaturing polyacrylamide gel with 5% glycerol, and run for 30 min at 80 W and subsequently for 7 h at 50 W and 25°C. The gel was transferred to a Whatman 3M paper, dried, and autoradiographed with 8 h exposure at 70°C on a Konica-AX X-ray film. The experiments were performed at least five times for each sample to match intensity by adjusting the concentration of template, and to check the reproducibility of the alterations as detected by the gains and losses of specific bands.

Reamplification and Cloning of PCR Products. Bands of similar mobility showing alterations in more than one tumor sample were excised from the polyacrylamide gel, and DNA was extracted by dissolving in 50 μl of Milli-Q H_2O. Five μl of the DNA was reamplified using the same conditions as above in a 50-μl reaction. The products were electrophoresed on 2% agarose gel and purified using DE-81 membrane. Forty ng of the above DNA was used in a 10-μl ligation reaction constituting 50 ng of pGEM-T vector (Promega) according to the manufacturer's instructions. Five μl of the reamplified products were electrophoresed on a 12% polyacrylamide gel, and the molecular size of each fragment was determined using an Alpha imager-ImageQuant system.

Sequencing of Clones. pGEM-T vector containing the appropriate insert was purified, 400–500 ng of the plasmid was used in a Big Dye (Applied Biosystems) cycle sequencing reaction using 3.2 pmol T7 primer according to the manufacturer's instructions, and capillary sequencing was performed on an ABI prism 310 Genetic Analyzer.

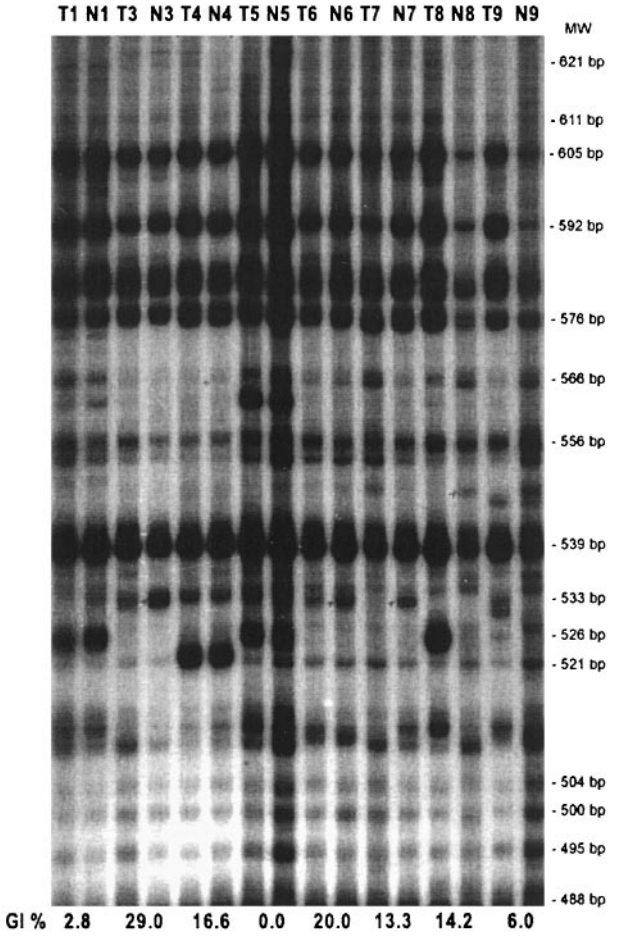

Fig. 1 Autoradiogram of ISSR-PCR products. The PCR was done using $(CA)_8RG$ primers, and the products were electrophoresed in 8% polyacrylamide gels. *T*, tumor DNA; *N*, corresponding normal DNA. Genomic instability (%) is shown below each pair of tumor and normal samples. Genomic instability was calculated by dividing the total number of bands generated in the normal sample by the number of altered bands.

Confirmation of LOH in 11p15 Region. Primers designed within the altered region of 11p15 (forward, 5′ CCG AGG AGT AAG AGC AGT AAG G 3′ and reverse, 5′ AAC ACA CAC ACA AGC ACC AC) and a sequence tagged site marker in this region WI-16945 (forward, 5′ TGT TGA TGG CTT CAA ATA CTT CA 3′ and reverse, 5′ CAG ATG TTA ATA CAC ACA GAC CTC G 3′) were used in a 10-μl multiplex PCR reaction containing 1× PCR buffer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.2 μM each primer, and 0.5 units of TaqDNA polymerase (Perkin-Elmer) using 50 ng of DNA as a template.

The cycling conditions were 3 min initial denaturation at 94°C, 30 cycles of 30 s at 94°C, 55°C, and 72°C, followed by final extension for 7 min at 72°C on a Perkin-Elmer GeneAmp 2400 thermocycler.

RESULTS

Basik *et al.* (6) first used ISSR-PCR to quantitate genomic instability in sporadic colorectal cancer. Using primer based on repetitive DNA sequences, anchored at the 3′ end with unique sequences to prevent slippage, this technique produces amplification of those DNA sequences, typically <2 kb in size, which are present between relatively close, inverted primer-binding repeat sequences (6). When CA repeat based primers were used, approximately 35–40 PCR products were generated (Fig. 1), whereas using CT repeat-based primers, approximately 20–25 products were generated. A preliminary comparison of bands generated by amplification of tumor and the corresponding normal samples revealed that the profile generated by CA dinucleotide-based primer showed a greater number of bands than that of CT-based primers in accordance with the fact that CA repeats are more widely distributed in the genome ($\sim 7 \times 10^6$ copies) than CT ($\sim 3 \times 10^6$ copies) dinucleotide repeats.

Tumor-specific alterations were detected as gains, losses, and intensity changes in electrophoretic bands. It must be noted that these alterations are not limited to insertions or deletions between or in the primer binding sites; larger scale processes such as deletions of portions of chromosomes or of entire chromosomes can also eliminate the corresponding bands. Both gains and losses were seen in the OSCC cases with a slight preponderance of gains than losses when $(CA)_8RG$ primer was used (Fig. 2*a*). No significant correlation was seen between the extent of genomic alterations and the stage of the tumor, age of the patient, or location of the tumor (Table 1).

We determined genomic instability according to Basik *et al.* (6) by dividing the number of altered bands in the tumor by the total number of bands in the corresponding normal tissue sample (Fig. 2*b*). The median genomic instability index was determined to be 8%.

Selected altered fragments (Fig. 3) were cloned and sequenced, and the sequence data obtained (GenBank accession no. AF539907-AF539917) were subjected to sequence alignment by using the Basic Local Alignment Search Tool sequence comparison tools of the National Center for Biotechnology Information public database. Human genomic blast was initially performed to identify the chromosomal region that matched the sequence followed by the standard nucleotide-nucleotide BLAST to determine the identity of the sequence using known human gene sequences in the database (Table 2).

Loss of the chromosomal region 11p15 was observed in five altered bands, two of them from the same sample (T3) in different ISSR-PCR experiments. The chromosomal region 17q25 was found to be lost in three altered bands from different samples. In the case of 11p15 the loss has been found to occur within the gene coding for polypeptide-*N* acetygalactosaminyl transferase 9 (*UDP-GalNAc*). The fragment from the altered region in 3p14 was completely homologous to human RF-C activator1 homologue (*RAD 17*), a human and mouse homologue of *Schizosaccharomyces pombe rad17*, which is a cell cycle checkpoint control gene. This is the first instance where ISSR-PCR has detected regions within genes directly involved in cell cycle regulation and cancer.

A multiplex PCR analysis using primers within the 11p15 altered region and an STS marker (WI 16945) in the 11p15 region was devised, and an amplicon of the human protein phosphatase gene was used as a template control. Alterations were detected as absence of the amplicons in 15% (6 of 40) of the cases. One of the cases (T8) from the original ISSR-PCR 11p15 loss region showed no amplification in the multiplex PCR reaction, whereas the other three (T3, T14, and T15) were amplified indicating a prevalence of allelic imbalance rather than LOH of this region in these cases (data not shown).

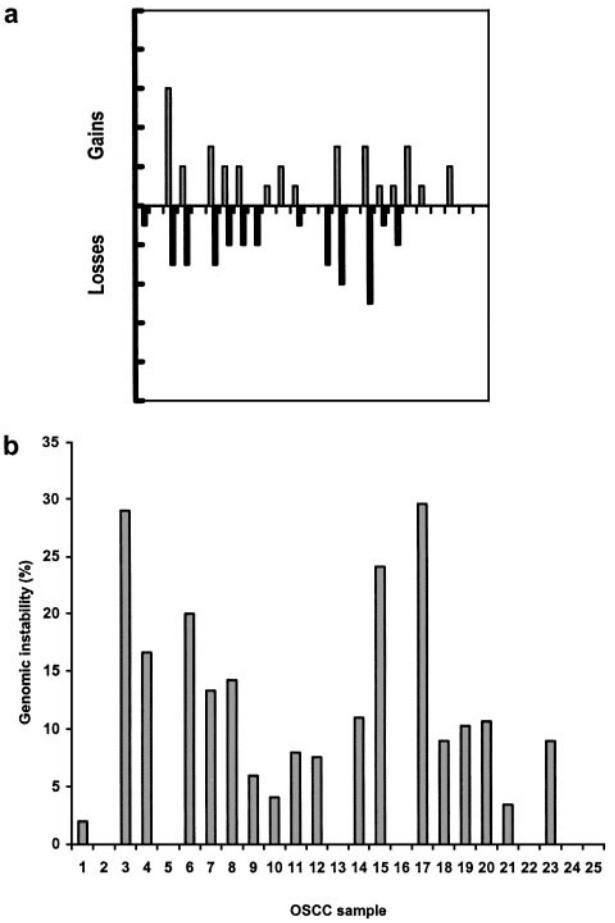

Fig. 2 *a*, evaluation of losses and gains in DNA bands generated by $(CA)_8RG$ primer. The chart shows gains and losses in the 25 samples studied with a slight preponderance of gains over losses. When gain/loss in not present in a sample, 0 value of gain or loss generates an apparent spacing on the *X* axis. *b*, assessment of genomic instability. Genomic instability calculated by dividing the total number of bands generated in the normal sample by total number of alterations in the 25 samples studied is depicted in the histogram. When no alterations are seen, the 0 value genetic instability generates an apparent spacing in the *X* axis.

DISCUSSION

We have used the ISSR-PCR developed recently to investigate genomic alterations in OSCCs. The banding profile generated by ISSR-PCR was found to be reproducible and reliable. Our observations prove that genomic instability is prevalent to a large extent in OSCC (median = 8%) and is important if not essential in the progression of this tumor type.

Two major types of genetic instabilities have been de-

Table 1 TNM[a] stage and genomic instability index of the tumor samples used in the study

Sl. no.	Sample no.	Age	Sex	Site	TNM staging	Genomic instability (%)
1	T_1/N_1[b]	60	M	Cheek lt.[c]	$T_4N_2M_0$	2.8
2	T_2/N_2	48	M	Alveolus rt.	$T_4N_1M_0$	0
3	T_3/N_3	62	M	Angle of mouth rt.	$T_4N_1M_0$	29.0
4	T_4/N_4	61	M	Cheek rt.	$T_4N_0M_0$	16.6
5	T_5/N_5	60	F	Lip	$T_3N_2M_0$	0
6	T_6/N_6	21	M	Tongue rt.	$T_4N_2M_0$	20.0
7	T_7/N_7	60	M	Cheek rt.	$T_4N_1M_0$	13.3
8	T_8/N_8	45	F	Alveolus rt.	$T_4N_2M_0$	14.2
9	T_9/N_9	50	M	Alveolus rt.	Tx	6.0
10	T_{10}/N_{10}	50	M	Cheek rt.	$T_4N_2M_0$	4.1
11	T_{11}/N_{11}	52	M	Cheek rt.	$T_4N_1M_0$	8.0
12	T_{12}/N_{12}	50	F	Cheek lt.	$T_4N_2M_0$	7.6
13	T_{13}/N_{13}	55	F	Palate	$T_4N_2M_0$	0
14	T_{14}/N_{14}	40	F	Tongue	$T_4N_1M_0$	11.0
15	T_{15}/N_{15}	36	F	Cheek rt.	$T_2N_1M_0$	24.1
16	T_{16}/N_{16}	50	M	Lip	$T_2N_1M_0$	0
17	T_{17}/N_{17}	46	F	Alveolus rt.	$T_4N_1M_0$	29.6
18	T_{18}/N_{18}	60	M	Angle of mouth rt.	$T_4N_2M_0$	9.0
19	T_{19}/N_{19}	67	M	Cheek rt.	$T_2N_0M_0$	10.3
20	T_{20}/N_{20}	40	F	Cheek rt.	$T_4N_3M_0$	10.7
21	T_{21}/N_{21}	70	M	Alveolus lt.	$T_2N_0M_0$	3.5
22	T_{22}/N_{22}	45	F	Cheek rt.	$T_4N_1M_0$	0
23	T_{23}/N_{23}	40	F	Cheek rt.	$T_4N_1M_0$	9.0
24	T_{24}/N_{24}	55	M	Tongue	$T_4N_2M_0$	0
25	T_{25}/N_{25}	70	M	Cheek lt.	$T_3N_2M_0$	0

[a] TNM, Tumor-Node-Metastasis.
[b] T, tumor; N, normal.
[c] Lt., left; Rt., right.

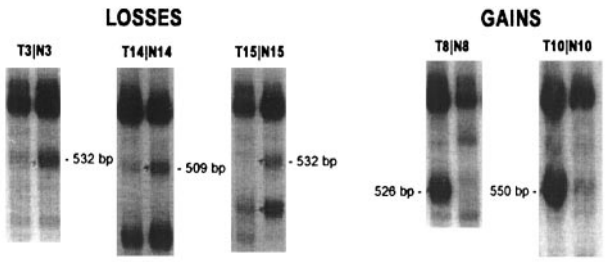

Fig. 3 Autoradiogram showing losses and gains in tumors. Regions of autoradiograms showing losses in three and gains in two tumors are depicted. The bands showing reproducible alterations in several samples were excised; the DNA was eluted, cloned, and sequenced to determine the chromosomal region altered.

scribed (13, 14), MIN, which arises because of a defective mismatch repair system and is characterized by widespread alterations in the microsatellite repeat regions (15), and CIN, arising because of mutations that affect the partitioning of chromosomes during mitosis characterized by large alterations involving thousands of nucleotide units in DNA (16).

It is known that occurrence of MIN phenotype is relatively low in OSCC and is evident by the fact that many LOH events rather than alterations in repeat regions (MIN) are observed by allelotyping of OSCC DNA with microsatellite markers (17–25). Reports of MIN from the Asian region show various degrees of incidence from nil (0%) to low (4–17%) to moderate (27–50%), whereas reports from developed regions of the world have consistently shown low incidence (0–7%); but in all of the studies reported LOH events occur more predominantly than MIN (17–25).

Studies done in our laboratory using four microsatellite markers in the chromosome region 10q22–26 using the same tumor samples with matched normal samples used for ISSR-PCR experiments also show that the occurrence of MIN of repeat sequences in OSCC is comparatively rarer than LOH events.[4]

The MIN phenotype is related to defects in the mismatch repair system. Loss of expression of the mismatch repair gene, *hMLH1*, predominantly by hypermethylation of the promoter region is associated with MIN. This is seen in many human cancer types including colorectal, pancreatic, and endometrial cancers (26–31). Studies done in our laboratory on incidence of hMLH1 methylation have shown this to be a rare event occurring in only 8% of the 99 samples studied (32).

Our ISSR-PCR data show that the other form of genomic instability, CIN phenotype (11), occurs to a greater extent in OSCC regardless of the tumor stage and its location, and is probably an early event in the progression of the disease. It has been reported that occurrence of MSI is independent of LOH events and of the alterations in ISSR-PCR profile. However, there is indeed a correlation between LOH events and degree of alterations in ISSR-PCR profile (12). In other words, the alterations detected by ISSR-PCR reflect the LOH events rather than MSI in the genome.

Recent studies have correlated the type of genomic instability (CIN/MIN) developing in a particular cancer to specific classes of carcinogens. It has been shown that alkylating car-

Table 2 BLAST results for the sequenced clones containing inserts of altered fragments from ISSR-PCR of oral tumor samples compared with normal tissue

Sl. no.	Fragment designation	Molecular weight (bp)	Clone	Alteration	Chromosomal region	GenBank accession no.	Product
1	Frag 18	606	T/N 9[a]	Loss	2p21	AF539915	—
2	Frag 12	544	T/N 6	Loss	3p14	AF539912	Homo sapiens RF-C/activator 1 homolog (RAD17)
3	Frag 19	623	T/N 12	Loss	3q28	AF539916	—
4	Frag 13	580	T/N 7	Loss	7p14	AF539913	—
5	Frag 2	532	T/N 15	Loss	7p15	AF539908	Hypothetical protein XP_088252
6	Frag 1	532	T/N 14	Loss	11p15	AF539907	Protein-UDP acetylgalactosaminyltransferase 9
7	Frag 3	532	T/N 3	Loss	11p15	AF539907	Protein-UDP acetylgalactosaminyltransferase 9
8	Frag 14	532	T/N 8	Loss	11p15	AF539907	Protein-UDP acetylgalactosaminyltransferase 9
9	Frag 16	532	T/N 3	Loss	11p15	AF539907	Protein-UDP acetylgalactosaminyltransferase 9
10	Frag 20	532	T/N 15	Loss	11p15	AF539907	Protein-UDP acetylgalactosaminyltransferase 9
11	Frag 8	529	T/N 12	Loss	11q14	AF539917	—
12	Frag 11	559	T/N 3	Loss	12p13	AF539911	KIAA1661 protein
13	Frag 6	526	T/N 8	Loss	12q22	AF539909	—
14	Frag 15	547	T/N 3	Loss	12q21	AF539914	L1 repeat, Tf subfamily, member 23
15	Frag 9	509	T/N 14	Loss	17q25	AF539910	—
16	Frag 10	532	T/N 15	Loss	17q25	AF539910	—
17	Frag 17	532	T/N 8	Loss	17q25	AF539910	—

[a] T, tumor; N, normal.

cinogens specifically give rise to tumors with the MIN phenotype, whereas bulky adduct-forming carcinogens lead to the CIN phenotype (33).

The nature of genomic instability now detected by the ISSR-PCR in OSCC, *i.e.*, CIN, is indicative of the fact that DNA adduct-forming carcinogens like benzo(*a*)pyrenes found in cured smokeless tobacco might play a major role in causing genomic instability rather than alkylating agents like *N*-nitrosamines, which lead to accumulation of mutations specific to G residues and are frequently associated with the MIN phenotype (34). Earlier observations from our laboratory indicated a comparatively low incidence of mutations in the tumor suppressor genes (3) that involve G residues (a preferential target of alkylating carcinogens). These findings support our hypothesis of the possibility of a major role of benzo(*a*)pyrenes apart from *N*-nitrosamines, thus far held responsible for the genesis of OSCCs.

Apart from measurement of genomic instability, the ISSR-PCR profile could also identify tumor-specific alterations in a given cancer type (10). We were biased in our selection of altered bands for cloning and subsequent sequencing. We looked for bands of similar mobility that were altered in more than one case with the aim to detect tumor-specific alterations that could lead to development of a potential marker region involved in OSCC. That genomic instability leads to loss of function of genes directly involved in the cell cycle regulation, and development of cancer is indicated by our results. Losses were observed in *UDP-GalNAc*, the expression of which was correlated directly to prognosis in colorectal cancer patients (35). One of the sample studied showed loss of the *S. pombe rad 17* gene homologue in humans. The yeast Rad 17 is involved in DNA repair/cell cycle checkpoint; the human homologue has the same functions and is a candidate tumor suppressor gene, and mutations in it can lead to cancers (36).

The reliability of the ISSR-PCR in detecting tumor-specific alterations has not been clearly demonstrated as yet. Sequencing results showing alterations in tumor-associated genes have not been reported, limiting the use of ISSR-PCR as a tool to quantify the extent of genomic instability only. Our results for the first time show the ability of ISSR-PCR to pick tumor-specific alterations showing homology to the coding regions of genes involved in the regulation of cell cycle and cancer development. This proves that ISSR-PCR is similar to CGH or perhaps an even more powerful technique, capable of detecting tumor-specific alterations in the genome, which might not just be random events caused by the CIN phenotype, but tumor- and region-specific targeted events evident by the fact that the same event has occurred in more than one tumor case. These alterations generated by the ISSR-PCR on further study have the potential to be developed into tumor-specific markers, and although no specific mutation could be absolutely diagnostic of a specific malignancy, the presence of an increased number of mutations indicated by the measurement of genomic instability index may indicate that cells were already on the path of neoplastic growth.

ACKNOWLEDGMENTS

We thank Anup Kumar Kesavan for help with the gel documentation. The sequencing facilities of the School of Biological Sciences and School of Biotechnology, Madurai Kamaraj University, were used for sequencing of the clones.

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