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Unusual Profile and High Prevalence of p53 Mutations in Esophageal Squamous Cell Carcinomas from Northern Iran[1]

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ABSTRACT

Over 15,000 human tumor p53 mutations have been recorded in the scientific literature, including over 700 mutations in esophageal tumors. There are no data on p53 mutations in esophageal cancer patients from Iran yet; however, this country experiences one of the highest cancer mortality rates in the world for esophageal squamous cell carcinomas (ESCCs). The causes of this high cancer burden in Iran remain obscure and do not appear to be related to tobacco and alcohol consumption, the two major risk factors identified in Europe and North America. Because molecular analysis of tumors can provide clues to endogenous or environmental factors contributing to high cancer risk, we examined 74 Iranian ESCCs for the presence of mutations in exons 5–8 of the *p53* gene by PCR and direct sequencing. Forty-eight of the 74 tumors (65%) had one or more *p53* gene point mutations, including 5 patients with two or more mutations and one with a tandem mutation in codon 242. Surprisingly, over one-third of the 54 mutations we identified were transitions at CpG sites (20 of a total of 54 mutations, or 37%), a class of mutation that is significantly less common (16% of mutations) in the compilation of ESCC mutations from other countries (χ^2 statistic, $P < 0.0002$), whereas transversions, which the literature shows to be common in ESCCs from non-Iranian patients, were infrequent in the tumors we examined here. Elevated levels of cyclooxygenase-2 and inducible nitric oxide synthase were observed in 74 and 91%, respectively, of tumors from Tehran as determined by immunohistochemistry, and high COX-2 expression correlated significantly with the presence of a p53 mutation in the tumor. Mediators of the inflammatory response in esophageal mucosa, perhaps in conjunction with specific dietary or cultural practices in Iran, may contribute importantly to the p53 mutation load in Iranian ESCC patients.

INTRODUCTION

p53 tumor mutations have been linked to specific carcinogen exposures, suggesting a molecular epidemiology approach to investigation of cancers for which the causes have remained elusive. Although it is well-established that tobacco and alcohol, particularly in combination, are the major causes of SCC in the United States and Europe, neither is thought to be important in the etiology of this disease in Iran, where both men and women are at elevated risk (1). Age-adjusted incidence figures of up to 171/100,000 have been reported in rural areas of northeastern Iran for ESCC,[3] the major histological subtype (2). The rates are >100-fold higher than in lowest incidence areas of the world, pointing to strong environmental and perhaps genetic influences. The precise risk factors responsible for the high prevalence of ESCC in northern Iran have remained a matter of conjecture. Cultural or environmental factors suggested to play a role include dietary vitamin and mineral deficiencies, mutagenic by-products of opiates, and consumption of regional specialties or scalding beverages, which are strong irritants to the esophageal mucosa (2–4).

We initiated a study to examine the p53 status in tumors of 74 ESCC patients from northern Iran, 40 from the capital city Tehran, and 34 from the Caspian Littoral, because no p53 mutation data were available in the literature on tumors from this region. Additional tumor tissue from paraffin blocks was available for 23 of the patients from Tehran, allowing immunohistochemical assessment of expression of COX-2 and iNOS, two enzymes known to be elevated in chronically inflamed tissues and in gastrointestinal tumors from other geographical areas.

MATERIALS AND METHODS

Tissue Collection and DNA Preparation. Formalin-fixed, paraffin-embedded esophageal tissue (surgically resected material or biopsy) from cancer patients diagnosed in Iran with ESCCs were collected for analysis. Informed consent was obtained from patients by participating scientists from Iran, and the study was approved by the Medical Ethics Committee, Ministry of Health, Iran as conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Hospital records were used to verify age, permanent residence, smoking history, and ethnicity of individuals. Archived histology sections were examined by the collaborating pathologist in Tehran (P. M.), and serial sections of 10-μm thickness were prepared for DNA extraction. Diagnosis of SCC was confirmed by the pathologist at the German Cancer Research Center, Heidelberg (H-J. G.), who designated an area of tissue material with >50% neoplastic cellularity for each specimen on H&E-stained slides, used to guide dissection for DNA extractions. Forty tumors were examined from patients who were long-term permanent residents of Tehran (most of whom were also born there) and for whom diagnosis of ESCC could be confirmed. Fixed specimens from 34 patients residing in the Caspian Littoral and who had undergone surgery in regional hospitals also were examined in the present study and subjected to the same scrutiny by the pathologists as the material from Tehran. Tissue areas with high (>50%) neoplastic cellularity were dissected from dewaxed slides, and the material was digested by proteinase K in SDS-containing buffer for 3–5 days at 50°C to release DNA suitable for PCR.

PCR and DNA Sequencing. Dewaxing, microdissection, DNA extraction, and PCR set-up were all performed in a special laboratory free of contamination from PCR products, with reagents and equipment reserved for these purposes as we have described previously (5). Each exon (exons 5–8) of the *p53* gene was individually amplified by a single 40-cycle PCR, using intron-specific 20-mer primers as described by Lehman *et al.* (6), and 27–30-mer primers described by the Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA). The longer primers efficiently generated PCR product from template DNA of formalin-fixed specimens that had proved difficult to amplify with 20-mer primers. PCR products were purified with Microcon 100 (Millipore) filters and sequenced directly by BigDye fluorescent dye dideoxy sequencing and microcapillary electrophoresis with an ABI 310 Genetic Analyzer according to the supplier's instructions (Applied Biosystems International). All samples with mutations were verified by two independent cycle sequencing PCR reactions and analysis of both DNA strands. In addition, of the 48 mutation-bearing tumor samples, 45 (94%) were reanalyzed by retrieving genomic DNA stocks, performing new PCR amplifications, and resequenc-

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[3] The abbreviations used are: ESCC, esophageal squamous cell carcinomas; ADC, adenocarcinoma; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IHC, immunohistochemistry; PCR, polymerase chain reaction.

ing of new PCR amplification products. (No material was left for the remaining three samples.) Finally, 9 tumors, in which a common (hotspot) mutation had been identified, were independently examined a third time, by preparing new tissue sections for microdissection, extraction of genomic DNA, amplification by PCR, and a third repeat of the mutation analysis. All confirmation experiments performed produced the same result as the first analysis.

Immunohistochemistry. Of the 74 ESCCs examined in this study for mutations, paraffin blocks of 23 tumors (all from residents of Tehran) were available for preparation of additional histology sections suitable for immunohistochemistry. Tissue sections (3 μm) were applied to precoated glass slides, and after dewaxing, were subjected to microwave antigen retrieval in citrate buffer. To block nonspecific reactivity and staining from endogenous peroxidase, sections were incubated in hydrogen peroxide (1%) for 15 min and in serum-free Protein Block (Dako, Inc.) for 5 min. After rinsing, slides were incubated overnight at 4°C with primary antibody, either polyclonal anti-COX-2 antiserum (Santa Cruz, dilution 1:200) or anti-iNOS monoclonal antibody (N32020; Transduction Laboratories; dilution, 1:100). Incubation with appropriate biotinylated secondary antibody was for 30 min, followed by incubation in avidin-biotin-peroxidase reagents (Vectastain ABC kit; Vector Laboratories), and visualization with 3,3′-diaminobenzidine (DAB kit; Vector Laboratories). Esophageal tumor cell lines TE-1 and TE-11 (7) served as positive and negative controls, respectively, for COX-2 expression in immunohistochemistry studies. The specificity of the COX-2 primary antibody was tested by incubating sections with primary antibody preabsorbed with the appropriate blocking peptide (C-20 P; Santa Cruz Biotechnology), which effectively blocked immunostaining of the COX-2-expressing cell line TE-11. Specificity also has been shown by Western blot analysis (8). Negative controls in which the respective primary antibody was omitted were also performed. Assessment of relative expression levels (0–3) was achieved according to a composite scoring system of staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) and the percentage of positive cells (0, 0–5%; 1, 6–25%; 2, 26–50%; 3, 51–100%) When the two components of the score were in distinct categories, the composite score was rounded off to the lower value. Statistical analysis of p53 tumor mutation status and COX-2 or iNOS immunostaining was performed with Fisher's exact test.

RESULTS

Of the 74 ESCC specimens analyzed from Iranian patients, 40 from Tehran and 34 from townships near the Caspian Sea, a total of 54 mutations were detected in exons 5–8 of the *p53* gene. Forty-eight of the 74 cases (65%) harbored one or more *p53* gene point mutations, including 5 tumors with two and one with three base changes. Patient list and mutation data from analysis of cases from Tehran are given in Table 1 and from the Caspian Littoral in Table 2 and Fig. 1. Because the average age of patients, sex ratio, mutation prevalence, and mutation patterns were highly similar in the two patient groups from Iran, we combined these two sets of data in Table 3 for comparisons with data from the IARC database (9), which comprises data from many areas around the world but which still has no data from Iran. Most

Table 1 *p53 mutation analysis in ESCC patients from northern Iran, Tehran*

No.	Patient code	Age/Sex (yr)	Tumor diagnosis	p53 mutations		
				Codon	Base	Type
1	TH-F97	68/F	SCC	273	CGT→TGT C→T	m,[a] CpG
2	TH-F98	68/F	SCC		No mutation	NA
3	TH-F99	NA/F	SCC	213	CGA>TGA C→T	s, CpG
4	TH-F100	53/F	SCC	Intron 7	G→C	sp
5	TH-F101	57/M	SCC	196	CGA→TGA C→T	s,CpG
6	TH-F102	59/F	SCC		No mutation	NA
7	TH-F103	27/M	SCC		No mutation	NA
8	TH-F105	70/F	SCC	242	TGC→TTC G→T	m
9	TH-F106	64/F	SCC	203	GTG→TTG G→T	m
10	TH-F107	72/M	SCC	248	CGG→CAG G→A	m, CpG
11	TH-F109	64/F	SCC	141	TGC→TAC G→A	m
12	TH-F111	68/F	SCC	213	CGA→TGA C→T	s, CpG
13	TH-F112	65/M	SCC		No mutation	NA
14	TH-F113	76/M	SCC	237	ATG→ATT G→T	m
15	TH-F114	60/F	SCC	248	CGG→CAG G→A	m, CpG
16	TH-F115	63/M	SCC	258	GAA→AAA G→A	m
				282	CGG→TGG C→T	m, CpG
17	TH-F116	58/F	SCC	229	TGT→T del −2	Frameshift
18	TH-F117	49/F	SCC	248	CGG→TGG C→T	m, CpG
19	TH-F118	65/M	SCC	273	CGT→CAT G→A	m, CpG
20	TH-F119	71/M	SCC	Intron 5	G→A	sp
21	TH-F120	58/F	SCC		No mutation	NA
22	TH-F121	68/M	SCC		No mutation	NA
23	TH-F123	51/F	SCC	Intron 6	G→T	sp
24	TH-F125	66/F	SCC		No mutation	NA
25	TH-F126	70/F	SCC	251	ATC→AAC T→A	m
26	TH-F127	65/F	SCC		No mutation	NA
27	TH-F128	74/F	SCC	212	TTT→TT del −1	Frameshift
28	TH-F129	64/M	SCC	213	CGA→TGA C→T	s, CpG
29	TH-F130	42/M	SCC		No mutation	NA
30	TH-F131	60/F	SCC	168	CAC→CGC A→G	m
31	TH-F132	27/F	SCC	296	CAC→TAC C→T	m
32	TH-F133	29/M	SCC		No mutation	NA
33	TH-F134	62/F	SCC	196	CGA→TGA C→T	m, CpG
				147	GTT→GTTGGGT ins+4	Frameshift
34	TH-F135	58/F	SCC	191	CCT→TCT C→T	m
				302	GGG→GAG G→A	m
				306	CGA→TGA C→T	s, CpG
35	TH-F136	62/M	SCC	206	TTG→TAG T→A	s
				243	ATG→TG del−1	Frameshift
36	TH-F137	66/F	SCC		No mutation	NA
37	TH-F139	40/F	SCC		No mutation	NA
38	TH-F140	66/F	SCC		No mutation	NA
39	TH-F 141	74/F	SCC	Intron 5	T→C	sp
40	TH-F142	52/M	SCC	286	GAA→AAA G→A	m

[a] m, missense; del, deletion; ins, insertion; s, stop mutation; sp, splice mutation; CpG, CpG transition; NA, not available.

Table 2 *p53 mutation analysis in ESCC patients from northern Iran, Caspian Littoral*

No.	Patient code	Age/Sex (yr)	Tumor diagnosis	p53 mutations			
				Codon	Base	Type	
1	Sa-F3	58/F	SCC	209	AGA→A	del[a]-2	Frameshift
2	Sa-F4	69/M	SCC	267	CGG→CG	del-1	Frameshift
3	Sa-F5	71/M	SCC	286	GAA→AAA	G→A	m
4	Sa-F6	60/F	SCC		No mutation		NA
5	Sa-F7	58/M	SCC	248	CGG→TGG	C→T	m, CpG
6	Sa-F8	80/M	SCC		No mutation		NA
7	Polsen-F11n	54/M	SCC	265	CTG→CGG	T→G	m
8	Nos-F12	50/M	SCC	152	CCG→CTG	C→T	m, CpG
9	Sa-F16	64/M	SCC	254	ATC→AT	del-1	Frameshift
10	Ghon-F23	70/F	SCC	248	CGG→TGG	C→T	m, CpG
11	Ghon-F24	64/F	SCC		No mutation		NA
12	Part-F26	50/F	SCC	153	CCC→CTC	C→T	m
				161	GCC→GTC	C→T	m
13	Nek-F31	74/M	SCC		No mutation		NA
14	Sa-F33	57/M	SCC		No mutation		NA
15	Sa-F38	69/F	SCC		No mutation		NA
16	Nos-F13	48/F	SCC	152	CCC→CTG	C→T	m, CpG
17	Sa-F41	79/F	SCC	178	CAC→CAAC	ins+1	Frameshift
18	Ba-F51	55/M	SCC		No mutation		NA
19	Sav-F53	50/F	SCC	213	CGA→TGA	C>T	m, CpG
20	Gham-F54	72/M	SCC		No mutation		NA
21	Ba-F56	70/M	SCC		No mutation		NA
22	Ba-F57	77/F	SCC	Intron 7		G→T	sp
23	Ami-F58	60/M	SCC	289–292		del-8	Frameshift
					CTCCGGAAGAAA→CTAA		
24	Sa-F67	66/M	SCC	248	CGG→TGG	C→T	m, CpG
25	Sa-F69	60/F	SCC	242	TGC→TTT	G→T	m
						C→T	m
26	Rod-F75	69/F	SCC		No mutation		NA
27	Gham-F76	51/F	SCC	155	ACC→ATC	C→T	m
28	Aghg-F85	53/M	SCC	169	ATC→ACG	T→C	m
29	Alia-F86	45/F	SCC		No mutation		NA
30	Gorg-F87	80/M	SCC		No mutation		NA
31	Khnb-F91	42/M	SCC	127–134	TCC . . . TTT→TT	del-22	Frameshift
					del.CCCCTGCCCTCAACAAGATGTT		
32	Gorg-F92	41/M	SCC		No mutation		NA
33	Sa-FII	38/F	SCC	248	CGG→TGG	C→T	m,CpG
34	Sa-FIII	72/M	SCC	213	CGA→TGA	C→T	m,CpG

[a] del, deletion; ins, insertion; sp, splice mutation; m, missense; CpG, CpG transition; NA, not available.

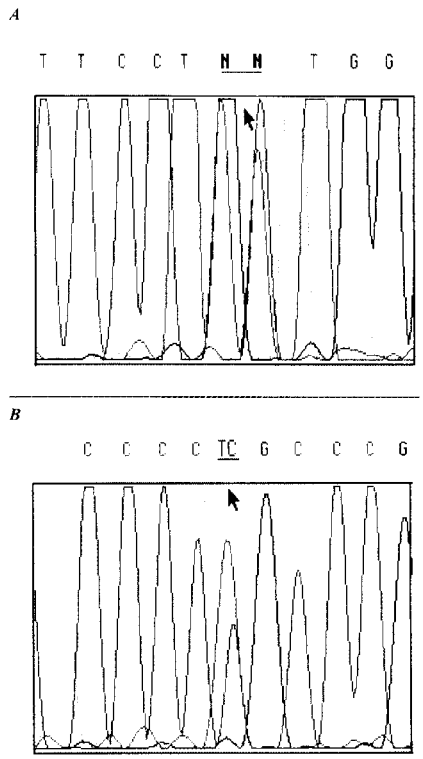

Fig. 1. Electropherogram of DNA sequencing (5′→3′) showing base substitution mutations in ESCC. *A*, a tandem mutation at codon 242 (TGC→TTT) in tumor Sa-F69 (see also Table 2). *B*, a transition mutation at codon 152 (CCG→CTG) found in tumor Nos-F12 (Table 2).

mutations we identified in Iranian patients were base substitutions (81% substitutions and 19% frameshifts), among which transitions at CpG dinucleotides were the most common (20 mutations of 54 total, or 37%), followed by transitions at non-CpG sequences (27%), whereas transversions were relatively infrequent (19%; Table 3). p53 sequence sites, where a mutation was found in >3 tumors, were two CpG dinucleotides identified as mutation hotspots in analyses of other cancer types with a high CpG transition frequency: codons 248 (7 tumor mutations), and 213 (5 mutations). The Iranian ESCC mutation profile is thus clearly significantly different from the esophageal SCC mutation spectrum in the IARC database, both with respect to the high prevalence of CpG transitions (37% *versus* 16%; χ^2 statistic, $P < 0.0002$) and the low prevalence of transversions (19% *versus* 41%; $P < 0.002$; Table 3).

There were more women ($n = 41$) than men ($n = 33$) among the 74 cases we had available for this study, reflecting the particularly high ESCC incidence rates for women in Iran, in marked contrast to many other high-risk areas of the world, where men are at vastly greater risk for this cancer (1). No significant differences between men and women in overall mutation prevalence or pattern were observed in our study, although because of the number of cases at our disposal, only very sharp differences would be expected to appear.

IHC was performed with 23 tumor specimens from Tehran to assess levels of COX-2 and iNOS (Table 4; Fig. 2; and data not shown), two enzymes associated with inflammatory reactions and recently found to be elevated in various types of gastrointestinal tumors (10–13). Tumor paraffin blocks for preparation of 3-μm sections on precoated slides for IHC were not available for the remaining patients. Seventy-four % of tumors examined by IHC (17 of 23) were immunoreactive

Table 3 *Summary of mutation data*

Test of significance: χ^2 statistic. Transitions at CpG sites and transversions of all Iranian patients are compared against these mutation classes in ESCC recorded in the database, which at present contains no data from Iran.

	No. of patients					Transitions			
	M	F	Total	Patients with mutation[a]	No. of p53 mutations identified	At CpG sites	Non-CpG sites	Transversions	Frameshifts
A. Iran									
Tehran	14	26	40	27 (67%)	32	12 (38%)	9 (28%)	7 (22%)	4 (13%)
Caspian Littoral	19	15	34	21 (62%)	22[b]	8 (36%)	6 (27%)	3 (14%)	6 (27%)
Total	33	41	74	48	54	20 (37%)	15 (28%)	10 (19%)	10 (19%)
B. IARC database									
					430	68 (16%) $P < 0.0002$	107 (25%)	180 (41%) $P < 0.002$	75 (18%)

[a] Three patients had more than one mutation.
[b] Includes one CT to GC tandem mutation.

Table 4 *COX-2 expression in 23 ESCC from Iran determined by immunohistochemistry*

	COX-2 composite staining score[a]			
	0	1	2	3
Tumors with p53 mutation[b] ($n = 15$)	0	0	4	11
Tumors without p53 mutation[b] ($n = 8$)	6	0	2	0

[a] Composite score of staining intensity and percentage of positively stained tumor cells (see 'Materials and Methods').
[b] Correlation of p53 tumor mutation with COX-2 expression, $P < 0.0005$, Fisher's exact test.

in the COX-2 assay, and 91% were positive for iNOS expression. All of the p53 mutation-bearing specimens (15 of the 23 tumors examined for COX-2 by IHC) revealed moderate to strong cytoplasmic immunoreactivity against anti-COX-2 antibody in tumor cells, whereas neoplastic cells in most of the samples in which no p53 mutations were detected were scored negative with COX-2 IHC (6 of 8; Table 4; $P < 0.0005$, Fisher's exact test). Reaction of inflammatory mononuclear infiltrating cells, known to express the enzyme, was also observed with COX-2 antibody. Although the sample size is small, this preliminary correlation is consistent with reports on inhibition of COX-2 expression by functional, nonmutated p53 (14) and on the association of p53 immunoreactivity with high levels of COX-2 expression in colorectal cancers (15).

DISCUSSION

Approximately two-thirds of the ESCC patients in our study harbored a p53 mutation, a prevalence that is comparable with patients from other high-risk areas such as Normandy (16) and in contrast to the modest prevalence (less than one-fourth of tumors with p53 mutations) from ESCC patients not identified as belonging to a high-risk group (17). Unlike the ESCCs from northern Iran that we examined, ESCCs from Northern American and European patients (the majority of whom smoke tobacco and consume alcoholic beverages) are characterized by comparatively low numbers of *p53* gene CpG transitions and a relatively high proportion of specific, strand-biased transversions (Ref. 9 and Table 3). This profile may reflect the mutagenicity of tobacco-derived compounds and the DNA repair-inhibiting activity of ethanol metabolites (18, 19). In keeping with this proposal, ESCCs of smoking and drinking patients in Europe and the United States are far more likely to harbor a p53 mutation than tumors of nonsmokers and nondrinkers, corroborating the notion that the named risk factors contribute importantly to mutation load in these populations (17), an observation that has been extended recently to lung cancer (20). In contrast, adenocarcinoma of the esophagus, a cancer thus far not closely linked to tobacco and alcohol consumption, is characterized by a high frequency of transitions at CpG sites (>50%) and low numbers of transversions (9, 21).

The high prevalence of p53 CpG transitions in Iranian SCCs of the esophagus we report here was thus unexpected and challenges the generality that CpG transition mutation prevalence is typical of adenocarcinoma but not SCC. Transitions at CpG sites can arise spontaneously by deamination of 5-methylcytosine to thymine but also may be provoked by various mechanisms including factors that cause chronic inflammation or high nitric oxide levels (22, 23). The frequent iNOS and COX-2 immunoreactivity among Iranian ESCCs is in

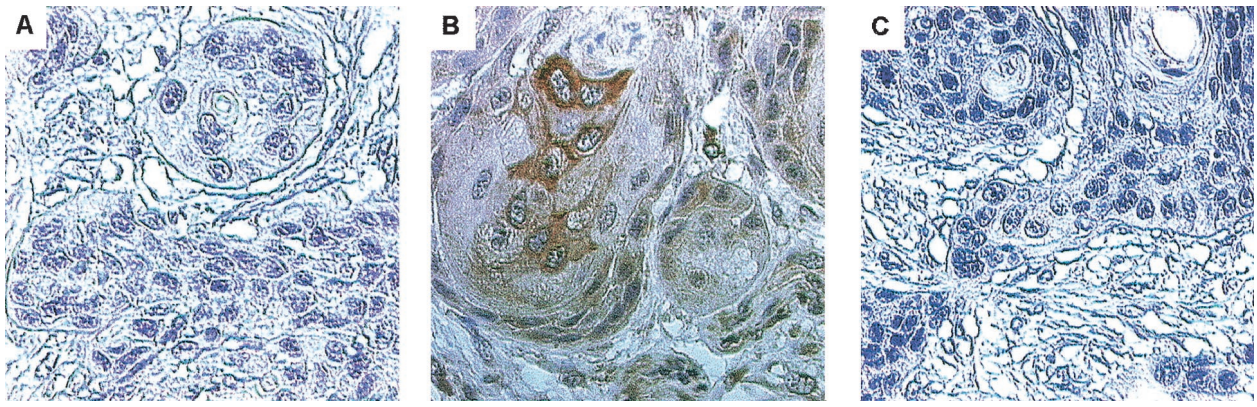

Fig. 2. Immunohistochemical staining for COX-2 in an ESCC exhibiting expression: *A*, control reaction without primary antibody (no immunostaining); *B*, with primary antibody, showing reactivity (brown cytoplasmic staining of some tumor cells); and *C*, specificity control incubated with blocking peptide and antibody (no immunostaining). Sections were counterstained with hematoxylin. ×200.

keeping with observations in gastrointestinal cancers from other geographical areas and risk groups, however, and is thus not *per se* a distinguishing feature of the patients in this study. The antiapoptotic, growth-promoting role of COX-2 has been attributed to its capacity to eliminate free cellular arachidonic acid and its capacity to generate growth- and angiogenesis-promoting prostaglandins. Future studies may address the issue of whether COX-2 and iNOS levels are consistently higher or deregulation of enzymatic activity more precocious in ESCC carcinogenesis in Iranian patients and thus more effective in generating transitions at 5-methylcytosine-guanine dinucleotides. Altered cytosine methyltransferase or G:T mismatch glycosylase activities (19, 24) and adduction at 5-meC:G bp are also factors that theoretically could increase mutation load at CpG sites (25); therefore, it would be interesting to consider what dietary factors and cultural practices, such as consumption of beverages at high temperatures, could influence CpG transition rate by one of these mechanisms. Synergistic interaction of these biochemical processes with deregulation of cell cycle control by dietary zinc deficiency in generating transition mutations would be another avenue to investigate experimentally (26–28), because this mineral deficiency has been implicated in the etiology of esophageal cancer in Iran.

The ethnic composition of the Iranian population includes people of both Persian ethnicity and the far less numerous Turkomans of the northeastern townships near the Caspian Sea, such as Gonbad. All 40 patients in our study from Tehran were of Persian ethnicity as well as most patients from the Caspial Littoral participating in this study; only 4 patients, all residents of Gonbad, were of Turkoman (Mongolian; Table 2) origin. We thus were unable to address the important question of p53 mutation frequency/patterns in relation to genetic susceptibility/background. The public health problem that ESCC poses in Iran is particularly acute among the Turkomans of Gonbad, where incidence figures are among the highest recorded in the world (2, 29). In this and other rural areas of the eastern Caspian Littoral, dietary supplements to food staples low in vitamin and trace elements might attenuate the cancer-promoting effects of these deficiencies.

Investigations on the endogenous/environmental factors that can be linked to the presence of p53 mutations, especially CpG transitions, may point to preventive measures that could be undertaken. Our initial study of 74 Iranian ESCC patients calls for further work in this population, including design and implementation of a molecular epidemiology-oriented program of tumor analysis.

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