Association between GSTM1 and CYP1A1 polymorphisms and survival in oral cancer patients

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Aims. Cancer patient's inherited genotype may influence his or her survival, but evidence for the role of these genetic differences in oral cancer survival has not yet been explored.
Methods. The authors evaluated polymorphisms in the GSTM1 and CYP1A1 genes for associations with overall survival in 100 oral squamous cell carcinoma (OSCC) treated patients and 100 controls who were followed up for survival within 2 years of the date of completion of their treatment. Overall survival was evaluated in Kaplan-Meier survival functions and Cox proportional hazards models.
Results. After adjustment for stage and histology, GSTM1null genotype was associated with shorter survival among OSCC patients, compared with GSTM1 present genotype. There was no association between CYP1A1 C genotype and survival in the overall study population.
Conclusion. The study indicated a potential role for GSTM1 polymorphism in predicting the clinical outcomes of treated oral carcinoma patients.

Key words: glutathione S transferase, cytochrome P450, polymorphism, oral squamous cell carcinoma, survival

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common cancer in males in India and is the third most common cancer in Indian females[1]. The overall survival has not changed in recent years, despite extensive research on the biological and molecular features of OSCC. Drug metabolizing enzymes (DME) are responsible for the metabolism of many of the exogenous chemicals that are toxic and carcinogenic. These include phase I enzymes (cytochrome P450 (CYP) enzymes) which helps in bioactivation of carcinogens and phase II enzymes (glutathione S-transferase (GST)) which play role in detoxification of electrophilic compounds formed by the phase I enzymes. Hence, the toxic effect of exposure, absorption and detoxification of carcinogens depends on a delicate balance between the phase I and phase II enzymes[2].

Despite the fact that various factors determine the efficacy of cancer treatment, systemic drug levels and tissue exposure to drug metabolites is determined by genetic differences in drug metabolizing enzyme activity of an individual[3,4]. CYP1A1 is known to be present in many epithelial tissues and activates tobacco procarcinogens like poly aromatic hydrocarbons and aromatic amines into their carcinogenic forms. The CYP1A1 MspI polymorphism has been associated with higher risk of tobacco-related cancers, such as oral and lung cancers[5]. Preliminary studies indicate that CYP1A1 is also involved in the metabolism of 5-fluorouracil, used routinely in treatment of oral cancer[6].

The GST family detoxifies carcinogens, reactive oxygen species and lipid peroxidation products, yielding excretable hydrophilic metabolites[7]. Lack of these enzymes may potentially increase susceptibility to various cancers because of a decreased ability to detoxify carcinogens[5,8]. Several chemotherapeutic drugs and their metabolites undergo the glutathione-dependent detoxification catalyzed by GST enzymes[9,10]. Polymorphisms of certain GST enzymes may alter the metabolism of chemotherapeutic drugs and modify the effectiveness of therapy, thus can predict differences in outcomes of treatment for cancers of various sites like breast cancer[11-13], leukemias[14-16] and colorectal cancer[17]. GST polymorphisms may thus influence survival in cancer treated patients. Two hypothetical reasons were suggested for GST polymorphisms and survival: (a) several chemotherapy agents are substrates for glutathione conjugation catalyzed by GST enzymes[9,16,18-21], as are cellular by-products of reactive oxygen damage[22,23], therefore, patients may differ in response to chemo and radiation therapy depending on GST activity; and (b) susceptible GST genotypes are associated with characteristic patterns of somatic changes in tumor tissue (e.g. p53 or K-ras mutations) (ref.[24-27]). If patients who have genotypes with low GST enzyme activity are expected to

have certain somatic changes, and if the somatic changes correspond to more aggressive tumor phenotypes[28], GST genotype may show association with survival differences. Various studies have reported the relation of GST polymorphisms and survival of lung cancer patients, with conflicting results[29-32]. Goto et al.[33] reported that lung cancer patients with GSTM1 null genotype had shorter survival than patients with GSTM1 present genotype. Thus, there are probable mechanisms through which inherited GSTM1 genotype of a cancer patient may influence his or her survival[34], but evidence for a role of these genetic differences in OSCC survival is not yet explored. To the best of our knowledge, there have been few studies that have examined the impact of CYP1A1 and GSTM1 polymorphism on host susceptibility to oral cancer[5,35,36] but ours is the first study examining these polymorphisms as a determinant for survival in OSCC treated patients. Associations between drug metabolizing enzyme polymorphisms and patient prognosis could contribute to identifying individual differences in their potential to benefit from particular types of therapy.

MATERIALS AND METHODS

Selection of cases and controls

For the present study histopathologically confirmed 100 post treatment cases of OSCC and 100 control subjects were selected. Eligible patients to be included in the post treatment group were identified from the Karnataka Cancer Therapy And Research Institute, Padmashree Dr. R.B. Patil Cancer Hospital, Navanagar, Hubli, and obtained information on pathologic and clinical variables. Institutional review board and ethical committee approval was obtained prior to the start of the study. The Padmashree Dr. R.B. Patil Cancer Hospital actively conducts annual follow-up for each patient, and maintains information on the date of last contact, vital status, and recurrence status. Patients had been followed up for survival within 2 years of the date of completion of their treatment and were queried for this study. Informed consent was taken from all participants. Information regarding tobacco habit, stage at diagnosis, tumor site, histology and type of therapy were obtained. Eligibility criteria included histopathological diagnosis of, primary well differentiated squamous cell carcinoma and treated with surgical resection leaving negative margins followed by chemotherapy and radiation therapy. The majority of patients who received chemotherapy were treated with cysplatin based chemotherapy. All cases were patients with cancers of the oral cavity, i.e., buccal mucosa, alveolus, palate and tongue.

Controls enrolled in this study were matched for age, gender and tobacco habits. Control subjects included patients seen in the KLE VK Institute of Dental Sciences, Belgaum with conditions requiring dental treatment. The blood samples were subjected for genotype analysis.

Genotyping

Five milliliters of venous blood were collected from all study subjects in vacutainer tubes containing EDTA using aseptic measures. The blood was stored at -20 ^{o}C and transported in ice to the laboratory. DNA was extracted from peripheral blood lymphocytes by standard RNase and proteinase K treatment and phenol-chloroform extraction. DNA samples were stored at -20 °C until further reactions. DNA samples were evaluated for quantity by spectrophotometry (by NanoDrop ND-1000 spectrophotometer) and quality by a 1% agarose gel run. Polymerase chain reaction (PCR) and restriction fragment length polymorphism was performed using DNA samples to determine the polymorphic genotypes at CYP1A1 and GSTM1 loci. The reaction mixtures underwent the following incubations in Applied Biosystems 2720 thermal cycler: 1 cycle of 96 °C for 30 s, 30 cycles of 94 °C for 30 s, 56 °C for 40 s, and 72 °C for 30 s, followed by a final cycle of 7 min at 72 °C. Samples were electrophoresed on 2% native polyacrylamide gels, stained with ethidium bromide and examined over UV light (UV Transilluminator).

Genotypes were analyzed using PCR-based methods as described below.

CYP1A1: The CYP1A1 mutation found in the 3-flanking region was detected by PCR and RFLP analysis using the MspI restriction enzyme[37]. The DNA fragment was amplified using the following primers: 5'-CAG-TGAAGA-GGT-GTA-GCC-GCT-3' and 5'-TAG-GAG-TCT-TGTCTC-ATG-CCT-3'. After amplification, the PCR product was subjected to restriction digestion using MspI. The products were then separated by agarose gel (2% gel) electrophoresis. The CYP1A1 polymorphisms were classified as homozygous for m1/m1 (CYP1A1 A genotype which produced a 340-bp band), heterozygous for m1/m2 (CYP1A1 B genotype which produced 340, 200 and 140-bp bands), or homozygous for m2/m2 (CYP1A1 C genotype which produced 200 and 140-bp bands) alleles.

GSTM1: The GSTM1 genotype was detected after PCR amplification using primers for the GSTM1 gene[38] and the globin gene. The GSTM1 primers were 5'-CTG-CCC-TAC-TTGATT-GAT-GGG-3' and 5'-CTG-GAT-TGT-AGC-AGA-TCATGC-3'. The wild-type samples produced a band at 300 bp. In the variant samples, the GSTM1 gene was absent (GSTM1 null), and no band was observed.

Globin gene: A portion of the globin gene was amplified as a positive control, producing a 200-bp fragment. The following primers were used: 5'-GAA-GAG-CCA-AGG-ACA-GGTAC- 3'and 5'-GGT-GTC-TGT-TTG-AGG-TTG-CT-3'.

Statistical analysis:

Statistical analysis was performed using the SPSS software (version 16). Survival was calculated for each participant of post treated OSCC patient from the date of diagnosis until the date of death or the date of last follow-up contact. Initial analyses included the assessment of patient and tumor characteristics by GSTM1 and CYP1A1 genotypes, using chi-square analysis. Survival estimates were based on the Kaplan–Meier survival function, with statistical significance assessed using the log-rank test. Hazard ratios (HRs) and their 95% confidence intervals (95% CI) were calculated from a multivariate Cox proportional hazards model,with adjustment for categories

of stage at diagnosis as strata in the model and other prognostic characteristics like age, site, gender and histology as covariates. Overall relative risk of death associated with GSTM1 and CYP1A1 were estimated from this cox model, with adjusted potential cofounding variables.

RESULTS

A total of 100 post treated OSCC cases and 100 controls were entered into the study, of which 73.9% were males (Table 1). The mean age of the control group (49.9 years; range, 18-79) was identical to that of the case groups (51years; range, 32-70; Table 1). Older patients (age >50 years) were not significantly associated with poorer survival compared with younger patients. Maximum cases (46%) in study groups were from buccal mucosa.

Tobacco history before OSCC treatment revealed that most (78%) were chewers. To evaluate gene-tobacco interactions, the prevalence of CYP1A1 and GSTM1 were stratified by tobacco history. As expected, OSCC cases had a significantly higher frequency of tobacco consump-

Table 1. Distribution of study subjects according to CYP1A1 and GSTM1 genotypes according to different characteristics.

Genotype	Number of patients	Gender		Age at diagnosis (years)		Stage at diagnosis			Site			
		Male	Female	<=50	>50	Stage II	Stage III	Stage IV	Buccal mucosa	Tongue	Alveolus	Palate
CYP1A1												
A(m1m1)	60	42	18	38	22	6	22	32	36	4	10	10
B(m1m2)	30	16	14	12	18	0	4	26	6	2	18	4
C(m2m2)	10	6	4	6	4	2	2	6	4	0	4	2
Chi-square		1.2440		0.9720		4.8610			4.4460			
P-value		0.5368		0.6150		0.0880			0.2170			
GSTM1present	56	40	16	30	26	6	24	26	28	6	14	8
GSTM1null	38	20	18	22	16	2	2	34	14	0	18	6
Chi-square		1.7320		0.0860		9.4650			4.0101			
P-value		0.1881		0.7701		0.0090*			0.2601			

*Significant at 5% level of significance ($P<0.05$).
Results were not interpretable for 6 subjects for GSTM1 genotype.

Table 2. CYP1A1 and GSTM1 genotypes with respect to tobacco consumption.

Group	Genotype	No. of patients	Tobacco mean duration	Mean Frequency	Lifetime exposure
Post treatment group	CYP1A1				
	A(m1m1)	60	21.67±9.29	11.03±5.51	243.6
	B(m1m2)	30	20.47±10.23	10.00±4.12	229.6
	C(m2m2)	10	15.80±10.90	7.80±3.19	127.1
	GSTM1present	56	21.68±9.49	10.57±5.20	231.2
	GSTM1null	38	19.95±10.04	10.53±5.00	237.5
Total		100	20.72±9.69	10.40±4.96	227.7
Healthy control	CYP1A1				
	A(m1m1)	48	5.5±2.77	6.25±2.38	33.2
	B(m1m2)	46	6.29±5.75	5.04±2.49	27.9
	C(m2m2)	6	5.50±0.71	6.00±6.00	33.2
	GSTM1present	78	5.90±4.83	5.42±2.41	29
	GSTM1null	22	6.56±2.65	6.33±2.18	40.5
Total		100	5.88±4.39	5.66±2.44	30.7

Post treatment cases had a significantly higher frequency and duration of tobacco consumption ($P<0.0001$) as compared to controls. Further, on comparing genotype with nature and lifetime exposure of tobacco consumption, odds ratio was not found to be significant. Results were not interpretable for 6 subjects for GSTM1 genotype for post treatment group.

tion (P<0.00001) compared to controls. Similarly oral cancer patients had significantly higher duration of tobacco consumption than controls (P<0.0001; Table 2). Lifetime exposure was calculated among all tobacco users for both cases and controls (Lifetime exposure= Frequency of chewing events per day X duration in years or number of cigarettes/10 X duration in years). Lifetime exposure for tobacco consumption revealed that oral cancer patients were maximally exposed compared to controls. Further, on comparing genotype with nature and lifetime exposure of tobacco consumption, odds ratio was not found to be significant.

A total of 30 deaths were recorded during the follow-up period with cancer as the cause of death for 19, other causes known for 4 and unknown for 7. Follow-up among patients who were alive at the end of observation was 7-19 months. Follow-up was essentially complete to 19 months, with only 4 living subjects censored (lost to follow-up). 6 cases were recorded with recurrence of oral cancer who were alive and getting treated for the same. When genotype was compared with age group and site of primary lesion, CYP1A1 and GSTM1 genotype were not associated with age and site (P>0.05). Most cases of deaths in the post treatment group had primary lesion in alveolus (60%) followed by buccal mucosa (26.7%, Table 3). Most cases of deaths in the post treatment group were seen in stage IV (100%).

The distributions of CYP1A1 and GSTM1 genotypes according to patient characteristics are shown in Table 1. The Kaplan-Meier survival functions for overall survival by GSTM1 and CYP1A1 genotypes are presented in Figures 1, 2 and 3. The Relative Risk (RR) of death associated with GSTM1, and CYP1A1 genotypes are shown in Table 4.

Table 3. Analysis with respect of sites with deaths among post treated oral squamous cell carcinoma patients.

Site	No. of patients	No. of deaths
Buccal mucosa, n[%]	46[46]	8[26.7]
Tongue, n[%]	6[6]	0[0]
Alveolus, n[%]	32[32]	**18[60]**
Palate, n[%]	16[16]	4[13.3]
Total	100	30

GSTM1 polymorphisms

Of the 100 subjects genotyped for GSTM1, the results were not interpretable for 6 subjects who showed uninformative genotyping, so the GSTM1 analysis is based on 94 subjects. None of the missed subjects reported reccurences. An association between GSTM1 null genotype and increased risk of OSCC recurrence was present in this study population; there was also an association between GSTM1null genotypes with stage IV cancers. On the basis of the difference in the survival curves by GSTM1 genotype (Fig. 1 and 3) and on the log-rank test (Table 4), there was an indication that GSTM1 null individuals had shorter survival than GSTM1present subjects. The Kaplan-Meier estimates overall survival for GSTM1 present subjects and for GSTM1 null subjects. GSTM1 is also associated with stage at diagnosis (P<0.05). After adjustment for stage and histology, which were strong predictors of survival, GSTM1null genotype was associated with shorter survival among OSCC patients, with an adjusted death RR of 1.20, compared with GSTM1 present genotype. Additional adjustment for age, tumor site or history of a previous primary malignancy had essentially no effect on the Relative Risk. When the genotype was compared between post treatment group and control group, GSTM1 null genotype showed significant association with patients who died due to reccurence in the post treatment group.

CYP1A1 polymorphisms

Genotypes for CYP1A1were available for all 100 subjects. When we tested the relationship between CYP1A1 genotype polymorphism in all OSCC patients and the survival, no significant difference was observed between Kaplan-Meier survival curves.

GSTM1 and CYP1A1 polymorphisms

We conducted further analyses to explore whether the combination of GSTM1 and CYP1A1 is associated with

Table 4. Relative Risk for survival rates in CYP1A1 and GSTM1 among post treated oral squamous cell carcinoma patients.

Genotype	No. of Patients	No. of Deaths	Relative risk (RR)	95% Confidence Interval	P-value
GSTM1 present	56	4	1.01	0.61-1.67	0.0259*
GSTM1 null	38	26	**1.20**	0.52-2.76	
CYP1A1					0.9908
A(m1m1)	60	10	0.90	0.38-2.13	
B(m1m2)	30	18	0.90	0.29-2.81	
C(m2m2)	10	2	0.47	0.05-4.16	

P value according to log rank test.
*Significant at 5% level of significance (P<0.05).

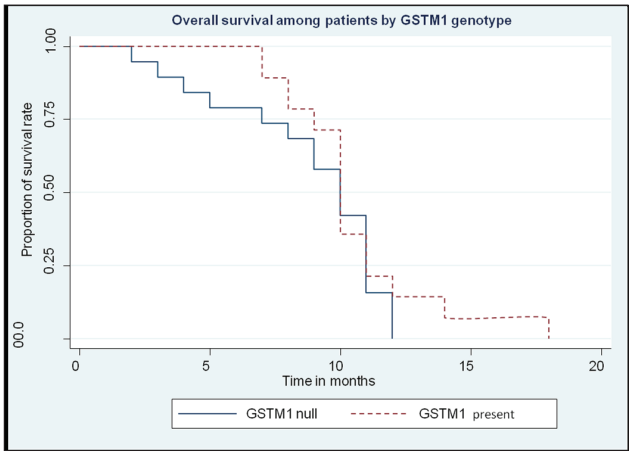

Fig. 1. Kaplan-Meier function for overall survival among patients treated for oral squamous cell carcinoma (SCC), by GSTM1 genotype.

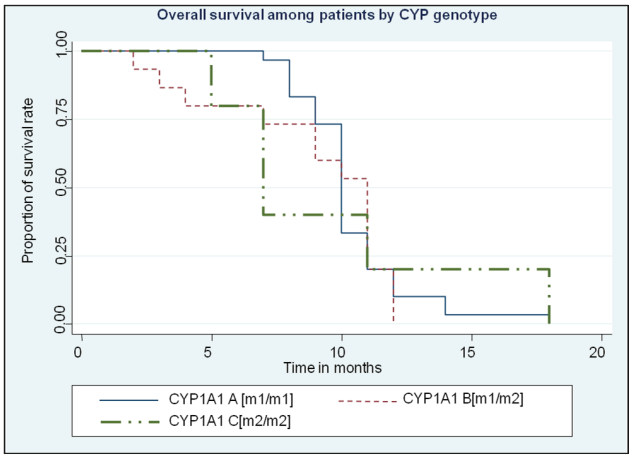

Fig. 2. Kaplan-Meier function for overall survival among patients treated for OSCC, by CYP1A1 genotype.

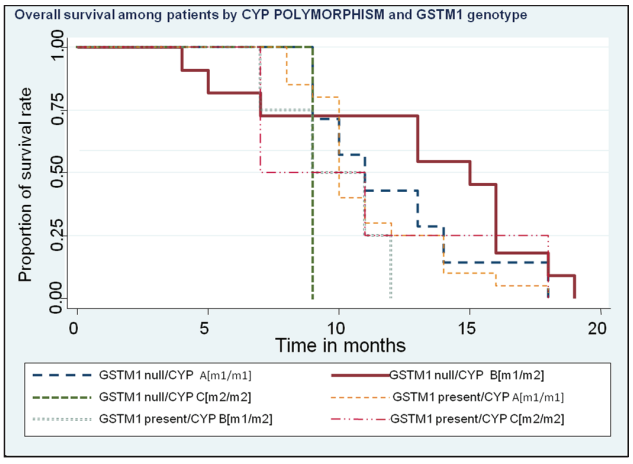

Fig. 3. Kaplan-Meier function for overall survival among patients treated for OSCC, by combined GSTM1 and CYP1A1 genotype.

survival. Patients who had both CYP1A1 B (m1m2) genotype and GSTM1 null genotype died the most compared to the other genotypes, but this did not reach statistical significance (data not shown).

DISCUSSION

Our analysis of the common polymorphisms of two genes namely CYP1A1 and GSTM1 in patients who were treated for oral squamous cell carcinoma demonstrates that subjects with the GSTM1 null genotype had more deaths than patients with GSTM1 present genotype. The relative risk of death associated with GSTM1 null genotype, adjusted for site, stage at diagnosis and histology, was 1.20 (95% CI 0.52-2.76).

The association of GSTM1 genotype with survival of cancer treated patients can be explained by the differences in detoxification of treatment agents or differences in carcinogen damage to DNA. Thus patients with GSTM1 null genotypes will have higher effective dose of chemotherapy leading to increased reactive oxidant damage to tumor tissue. Hence in GSTM1 null individuals therapy might be more effective, indicating longer survival as reported for GSTM1 null breast cancer patients[11,13].

In the present study of OSCC, the direction of the association was the reverse. Patients with reduced ability for GST-mediated detoxification showed shorter survival probably due to more severe therapy-related toxicity. This was consistent with a previous study in which GSTT1 null leukemia patients treated with high-dose therapy reported an increased number of deaths accredited to therapy-related toxicity[16]. The shorter survival in association with GSTM1 null genotype that was observed in the present study is also consistent with Goto et al.[33] who suggested that smokers with GSTM1 null genotype had more tendency to develop cancers with mutations of p53, K-ras, and/or genes that are known to play a role in tumor development, growth and metastasis. Thus GSTM1 null patients would be likely to have more aggressive tumor biology and shorter survival, even after taking into account stage at diagnosis, as was observed in the present study.

This hypothesis is further supported by a study which reported that patients with breast carcinoma and inheritance of double null deletion of GSTM1 may have an increased risk of a secondary chemotherapy-induced hematologic neoplasm[39]. Interestingly, Crump et al.[40] found a slightly higher prevalence of GSTM1 gene deletions in patients with secondary acute myeloblastic leukemia (AML) compared to patients with de novo AML or controls; however, their result was not statistically significant.

Vaury et al. hypothesized that CYP1A1 genoype and GSTM1 null interactions result in a greater-than-additive risk for DNA damage and cancer[41] Moreover, deletion of GSTM1 is associated with strong inducibility of CYP1A1 gene transcription by 2,4,7,8-tetrachlorodibenzo-para-dioxin, suggesting that this genotype combination predisposes to an increased risk for tobacco-associated DNA damage. However, in our study, no significant association was seen in the combined genotype of GSTM1 and CYP1A1 with

survival of patients. Although studies have shown association of CYP1A1 with incidence of oral cancer[42], no association was seen between this genotype and survival of OSCC in the present study population.

In the present study, most patients (78%) were tobacco chewers suggesting that the tobacco chewing habit with or without BQ prevalent in India also contributes to increased risk of oral cancer, due to additional exposure to alkaloids and polyphenols from the areca nut whereas in western countries, cigarette smoking and heavy alcohol consumption are the main risk factors[43]. In our study smokers constituted a low percentage of subjects, and therefore the risk due to smoking could not be seen. Next, estimating the cumulative tobacco dose for patients exhibiting MspI genotypes of CYP1A1, it was found that SCC patients with genotype C had a relatively lower dose than patients with genotypes A and B. Also, the estimated tobacco dose for the patients with GSTM1 null was less than that for GSTM1present patients. It was suspected that the genotypes C and GSTM1 null play an important role in individual differences in susceptibility to oral SCC, especially to the lowest tobacco dose level. The results of this study revealed that genetically predisposed BQ/tobacco chewers are much more susceptible to environmental and life-style risk factors.

Another interesting finding in the study was that though most cases were from the buccal mucosa (46%), most deaths in the post treatment group had the primary lesion in alveolus (60%) followed by buccal mucosa (26.7%). This is consistent with Tanimoto et al. who also reported that buccal mucosa and upper gingiva appear to be the most susceptible tissues for OSCC in patients carrying the combination of homozygous CYP1A1 (m1/m1) and GSTM1 (ref.[36]). Further, we also noted that most cases of deaths the in post treatment group were seen in stage IV (100%).

CONCLUSION

The underlying mechanism for the GSTM1-survival association should be further investigated. If the mechanism for the GSTM1-survival association is through the presence of p53 and/or K-ras mutations, as seems plausible, then the results of the present study may serve to reinforce the importance of considering GSTM1 genotype in future studies of oral cancer survival. Hence it is concluded that the xenobiotic metabolizing enzymes reported in the present study, GSTM1, significantly alter the prognosis of OSCC treated patients. The present study reinstates the complexity of the interplay between genetic factors as determinants of survival of OSCC. Knowledge of the prevalence and distribution of common genetic susceptibility factors and the ability to identify susceptible individuals or subgroups will have substantial preventive implications, in particular if more data are collected to show that people with certain "at risk" genotypes are more susceptible to recurrence after OSCC treatment.

CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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