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Association of *EGFR* gene polymorphism in head and neck cancer patients with tobacco and alcohol consuming habits

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Abstract
Single nucleotide polymorphisms (SNP's) in the epidermal growth factor receptor (EGFR) play a crucial role in head and neck cancer (HNC) disease progression and targeted therapies. Hence the present study aims to identify the mutations in *EGFR* gene (exon 20) in HNC considering their exposure to tobacco and alcohol habits. Mutational analysis was carried out by polymerase chain reaction (PCR) followed by single stranded confirmatory polymorphism (SSCP) techniques on the study group comprising of 129 HNC cases and 150 healthy volunteers. Four different SNP's (R776H, G779G, Q787Q, and L798H) were observed with the overall mutation rate of 75.19% in HNC cases and 46% in controls. Q787Q was found to be more prevalent ($p < 0.05$) and its genotypes GG, GA, and AA were 24.80%, 61.24%, and 13.95%. The study concluded that EGFR was found to be a polymorphic gene associated with HNC disease, and these SNPs were also prevalent in healthy volunteers with tobacco and alcohol habits.

Keywords: EGFR mutations; tyrosine kinase domain; head and neck cancer; ERBB receptor; squamous cell carcinoma.

Introduction

The human epidermal growth factor receptor (*EGFR*) gene codes for a 170 kDa transmembrane glycoprotein, plays a critical role in the signal transduction pathways for cell proliferation, differentiation, and survival (Grandis and Sok, 2004). A loss of constraints on the EGFR, and its signaling may result in deregulation of EGFR pathway and associated with neoplastic cell proliferation, migration, stromal invasion, resistance to apoptosis, and angiogenesis (Wu *et al.*, 2008; Olayioye *et al.*, 2000). Head and neck cancer (HNC) is the fifth most common cancer worldwide, with an estimated annual global incidence of over 500,000 cases (Parkin *et al.*, 2001). The most common form of malignancy is squamous cell carcinoma (SCC). Tobacco and alcohol are the individual risk factors for HNC and alcohol can potentiate the tobacco-related carcinogenesis (Decker and Goldstein, 1982). EGFR is over expressed in approximately 80% of head and neck squamous cell carcinoma (HNSCC). The over expression occurs early in the head and neck tumorogenesis and is associated with advanced stages of the disease and poor survival (Ang *et al.*, 2002).

EGFR is over expressed in a variety of human cancers including lung, colon, pancreas, breast, ovary, bladder and kidney, and gliomas (Zhang *et al*., 2010; Normanno *et al.*, 2006). Somatic mutations can get activated due to cellular environmental changes leading to single nucleotide polymorphisms (SNP's) which are responsible for the over expression of this tyrosine kinase (TK) family receptors. A number of mutations in EGFR have been observed in tumors where gene amplification has occurred (Jorissen *et al.*, 2003). Somatic mutations in exons (18–21) encoding the TK domain of the EGFR were reported in lung adenocarcinoma. Thus, EGFR become an attractive target for the development of cancer therapeutics, several targeted drugs were developed against TK domain. Gefitinib and erlotinib are active site tyrosine kinase inhibitors (TKI) of EGFR. Apart from causing over expression, some of these genetic lesions of EGFR influence the response to TKI.

Inframe deletions and L858R are associated with increased sensitivity of lung adenocarcinomas to the selective EGFR kinase inhibitors. Tumor cells obtained after disease progression contained a second site mutation involving a C–T change at nucleotide 2369 in exon 20, which substitutes methionine for threonine at position 790 (T790M) which showed resistance to EGFR inhibitors. This accounts for more than 50% of primarily EGFR – TKI sensitive lung tumors which become resistant to EGFR inhibitors. Other mutations which showed resistance to drugs were found in exon 19, such as D761Y and L747S, as reported (Kobayashi *et al.*, 2005; Balak *et al.*, 2006; Costa *et al.*, 2007); however, these mutations seem to be rare. In addition to non-small cell lung carcinoma (NSCLC), there was an evidence that gefitinib could be a potential agent for the treatment of other tumors, including HNSCC (Cohen *et al.*, 2003). Several reports have described that EGFR mutations in HNSCC patients are heterogeneous, show ethnic differences in the frequency of occurrence and vary from 7% in Asians to 0–4% in white patients (Van Damme *et al.*, 2010; Na *et al.*, 2007; Sheikh Ali *et al.*, 2008; Lemos-González *et al.*, 2007; Schwentner *et al.*, 2008).

EGFR mutations are inversely correlated with tobacco consumption and are reported to be more frequent in adenocarcinoma of women of Asian descent than in Caucasian (Shigematsu *et al.*, 2005). But the other studies showed that in the HNC, the EGFR mutation occurred in SCC and smokers (Lee *et al.*, 2005). Due to these various aspects of discrepancies on EGFR it is important to understand the association of SNP's with HNC and TKI. So the present study aims to determine the mutations prevalent in T_K domain (exon 20) of EGFR and its association with disease in correlation with habitual tobacco and alcohol users of South Indian population.

Patients and Methods

Study group: inclusion and exclusion criteria
Newly diagnosed 129 HNC tissue biopsies were collected from ENT hospitals and Mehdi Nawaj Jung Cancer Hospital, Hyderabad with informed consent. Pathologically confirmed HNC cases formed the patient (cases) group. Blood samples (2 ml) from 150 healthy age and sex matched volunteers were collected and these samples were considered as a control group. The patients under radiation and chemotherapy treatments were excluded.

The demographic details of the study group were collected using a structured questionnaire including age, sex, and personal history having smoking, alcohols, and tobacco chewing habits. Other details such as clinical history and primary cancer site were retrieved from medical records.

Sample processing for genotyping
In the present study after collecting patient and volunteer details (demographic and clinical records) mutations in *EGFR* gene were investigated in the study group consisting of randomly selected cases and controls as mentioned above.

DNA extraction: DNA was extracted from tissue biopsies and blood samples by ethanol precipitation protocol (Miller *et al.*, 1988). The quality and quantity of the DNA was analyzed by agarose gel electrophoresis and spectroscopy. The extracted pure DNA samples were stored at –20°C till further use.

Polymerase chain reaction (PCR) amplification of exon 20: The TK domain of EGFR flanking in the exons of 18–21 were selected for the study. The primers to amplify the whole region of exon 20 was selected to perform PCR with the primers: forward prime, 5′-CCATGCGAAGCCACACTGA-3′; reverse prime, 5′-CGTATCTCCCTTCCCTGATTACC-3′ (Willmore-Payne *et al*., 2006). The 248 bp product of exon 20 was amplified in a 50 µL reaction with 1× KCl buffer, 1.8 mM $MgCl_2$, 200 µM of each dNTP's, 1.5 U of Taq polymerase, 10 pm of each primer, and 100 µg of DNA template. The initial denaturation at 95°C for 5 min and then at 95°C for 40 sec, annealing at 60°C for 35 sec, extension at 72°C for 40 sec, and final extension at 72°C for 7 min were carried out for 35 cycles on eppendorf thermal cycler. The quality and specificity of the amplified exon 20 region was confirmed on the 2% agarose gel electrophoresis with 100 bp molecular marker.

Mutational analysis by single stranded confirmatory polymorphism (SSCP): Polyacrylamide gels were used to process the 248 bp PCR product for SNP analysis. The conversion of double stranded DNA into single stranded DNA was achieved by mixing 5 µL of the PCR product with 10 µL of denaturation buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, EDTA,

and NaOH) and the samples were denatured at 95°C for 5 min. The denatured samples were cooled immediately on ice for about 5 min. The processed samples were immediately loaded on 10% polyacrylamide gel electrophoresis and the run was carried out for 4 h at 150 volts. The gels were silver stained to observe the banding pattern of mobility shifts.

Sequencing: Samples with variant mobility shifts were processed for sequencing with Sanger's dideoxy method. The sequenced forward and reverse strands were further analyzed for SNPs. The sequence variations were confirmed with the help of wild type sequence. The samples were repeatedly processed to check the consistency and to confirm the SNP's found.

Statistical analysis

The statistical analysis was carried out with open EPI software version 2.3.1. The clinical characteristics were expressed as percentages. The Fisher's exact test was used to evaluate the association of HNC disease with the demographic parameters as appropriate. A two-tailed p-value was used in all the analysis and p-value < 0.05 was considered to be statistically significant.

Results

Patient characteristics

The 129 HNC cases were SCC and the median of the age was found to be 55 (20–85) years. The three categories of SCC includes well differentiated SCC (WDSCC), medium differentiated SCC (MDSCC), and poorly differentiated SCC (PDSCC). The male–female ratio was 1.01:1.87. In this group, 59% were habitual to tobacco and alcohol. Based on their habits to one or more habitual factors, cases were categorized. In the total group, 34.10% cases were smokers of bedi, chutta (forms of tobacco), and cigarette. Alcohol drinkers were 53.48% further these were habituated to whisky, sara, and kallu (various form of Indian alcohols). About 56%, 63 HNC cases had the habit of chewing of tobacco, pan, gutka, and khaini (some of these are Indian forms of tobacco). The demographic details are presented along with the mutational frequency of the *EGFR* gene (Tables 1 and 2).

Control group

A total of 150 healthy volunteers which formed the control group were 50 years of median age and the male–female ratio was 1:1. In control group, a total of 46% were habitual to tobacco and alcohol. Among them 16.66% had chewing habits, 31.33% were smokers, and 36.66% (55) were habituated to alcohol. The demographic details are presented along with the mutational frequency of *EGFR* gene (Tables 1 and 3).

SNP studies and analysis of exon 20

This was the first study on EGFR mutations in HNC from South Indian population. The study determined the frequency of EGFR exon 20 SNP's in association with disease state, gender, risk factors, and age. A total of four point-mutations were found in EGFR exon 20 (Table 1). The observed EGFR mutation frequency in HNC cases and controls was 75.19% and 46%. The prevalence of SNP's in exposure (smoking, alcohol, and chewing) and non-exposure groups of HNC cases and healthy controls were calculated. We found a significant association of SNP's with HNC cases of no habits ($p = 0.01$).

In smokers, the mutational frequencies was 82% in cases and 75% in controls. In alcohol habituals, the mutational frequency of SNPs was 75.36% in cases and 82% in controls. In chewing habituals, the mutational frequency was 74.39% in cases and 90% in controls. The age of the patients was also an important factor in considering the risk factors and mutations in

Table 1: SNP's of exon 20 in *EGFR* gene.

S. No.	Nucleotide change	Amino acid change	Mutant type	HNC cases ($n = 129$)	Controls ($n = 150$)	p-value
1	G2327A	R776H	Missense	31	19	0.02*
2	C2337T	G779G	Silent	31	19	0.02*
3	G2361A	Q787Q	Silent	97	69	0.01*
4	T2393A	L798H	Missense	31	19	0.02*

*Indicates $p < 0.05$.

Table 2: Demographic and clinical data of HNC cases with frequency of Q787Q genotype.

Demographic data cases	No. of cases (n = 129)	Frequency of mutations (%)
HNC cases	129	97 (75.19%)
Genders		
Male	65	47 (36.43%)
Female	64	50 (38.75%)
Median age	55	
≤55	67	50 (38.75%)
>55	62	47 (36.43%)
Habituated to		
No habits	16	13 (81.25%)
Smoking	7	5 (71.42%)
Alcohol	2	Nill (0%)
Chewing	35	25 (71.42%)
Smoking and alcohol	22	18 (81.81%)
Smoking and chewing	2	2 (100%)
Alcohol and chewing	31	22 (70.96%)
Smoking, chewing, and alcohol	14	12 (85.71%)
Tissue differentiation		
WDSCC	86	64 (49.61%)
MDSCC	32	25 (19.37%)
PDSCC	11	8 (6.20%)

Table 3: Demographic data of healthy volunteers with the distribution of Q787Q genotype.

Demographic data control group	No. of controls (n = 150)	Frequency of mutations (%)
Controls	150	69 (46%)
Genders		
Male	75	53 (35.33%)
Female	75	16 (10.66%)
Median age	50	
≤50	79	55 (36.66%)
≥50	71	14 (9.33%)
Habituated to		
No habits	84	18 (21.42%)
Smoking	5	4 (80%)
Alcohol	7	2 (28.57%)
Chewing	2	2 (100%)
Smoking and alcohol	34	26 (76.47%)
Smoking and chewing	1	Nill (0%)
Alcohol and chewing	10	10 (100%)
Smoking, chewing, and alcohol	7	7 (100%)

the *EGFR* gene. About 65.11%, (84) cases were in the range of 45–65 age groups and between these 34 female and 29 male cases had mutations. In controls, 25 males and 8 females were detected with mutations in the gene in this age group. The distribution of SNP frequencies among males and females are presented in Tables 2 and 3.

Q787Q genotype

A silent mutation of Q787Q was found to be prevalent in the study group. Transition of G–A at 2361 nucleotide resulted in three possible genotypes GG, GA, and AA (Figure 1). The mutational frequency of the genotypes is presented in Table 4. GA was found to be significant with HNC cases ($p > 0.05$).

Four SNP's R776H, G779G, Q787Q, and L798H were found with 24.3% in cases and 12.66% in controls (Figure 2). The combination of smoking and chewing of tobacco was also prevalent in these groups. The SNPs observed were significant with the disease state. Similarly, male and female frequencies were also found.

Figure 1: *EGFR* exon 20 gene sequence with Q787Q polymorphism. Q787Q: A transition of G to A at 2361 nucleotide creates a silent mutation at 787 amino acid on TK domain of *EGFR* gene.

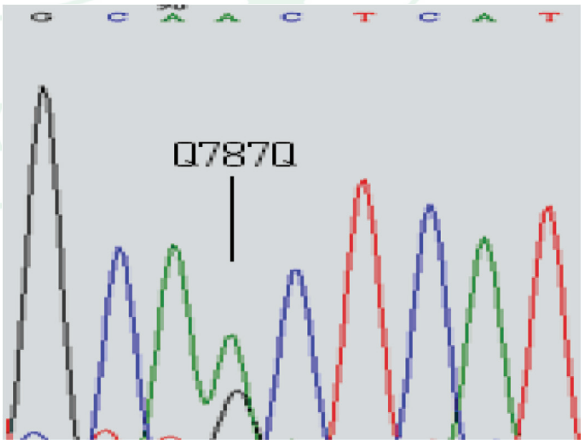
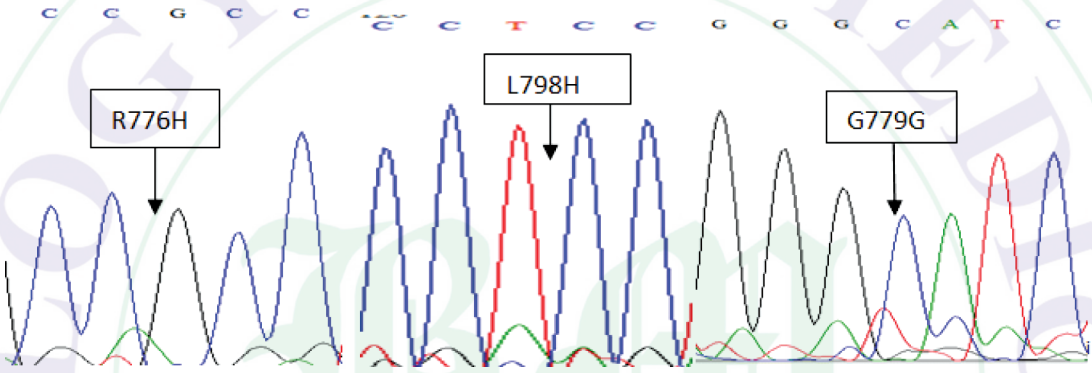


Table 4: The significant status of the frequency of (G2361A) Q787Q genotype.

Genotype	Cases (n = 129)	Controls (n = 150)	p-value
GG	32	81	$p < 0.05$
GA	79	55	$p < 0.05$
AA	18	14	$p < 0.05$

Figure 2: SNP in TK domain (exon 20) of *EGFR* gene. R776H: A transition of G to A at 2327 nucleotide results an missense mutation of amino acid from arginine to histidine at 776 amino acid position. L798H: A transition of T to A at 2393 nucleotide results an missense mutation of amino acid from leucine to histidine at 798 amino acid position. G779G: A transition of C to T at 2337 nucleotide results a silent mutation at 779 amino acid position.



Discussion

EGFR is the TK receptor of ERBB family, mutations in *EGFR* gene leads to upregulation of downstream signaling pathways and are associated with many cancers. Over expression and polymorphism affects the prognosis of the disease. Thus, EGFR becomes an attractive target to control the tumor cell proliferation. TKI like gefitinib and erlotinib are targeted to control the signaling of EGFR. As reported earlier, the role of mutated EGFR as a model to target drug molecules like gefitinib, erlotinib, and its analogs to inhibit the EGFR TK domain which is on the cytoplasmic side of the receptor and involved in drug target interactions (Kotra *et al.*, 2008). Consequently, several reports have appeared demonstrating the benefits of EGFR mutations in targeted therapies.

In this study, the prevalence of EGFR mutations in HNC patients and also in healthy volunteers with smoking, alcohol, and chewing habits were investigated. The mutational frequency observed among cases and controls was significant and showed an association with disease state. Very few studies were available on EGFR mutations in HNSCC. The literature showed that Asian population has high prevalence than other population (Van Damme *et al.*, 2010; Na *et al.*, 2007; Sheikh Ali *et al.*, 2008; Lemos-González *et al.*, 2007; Schwentner *et al.*, 2008). EGFR mutations were not reported from American, Austrian, and French studies in HNSCC (Lemos-González *et al.*, 2007) and in other studies it was observed to be 1–14% (Pühringer-Oppermann *et al.*, 2007; Willmore-Payne *et al.*, 2006; Guo *et al.*, 2006). In this study, it was relatively higher and supported that the ethnic variations might change the prevalence of mutations (Hsieh *et al.*, 2011).

HNC are amongst the commonest malignancy in India and globally, accounting for around 20% cancer burden in India. The consumption of tobacco and alcohol are the major 'preventable' risk factors (Siddiqui *et al.*, 2012). Conversely, a small portion of patients with HNC are non-smokers and non-drinkers (Mashberg *et al.*, 1993). The equal male–female ratio of the study emphasizes the association of exposed risk factors with the disease and genetic variations. Chewing of tobacco in females showed a higher frequency (51.54%) in inducing mutations

than in males (48.45%). Because of the tobacco chewing habits this study was not compatible with the ratios of male–female HNSCC (3:1 to 5:1) as in other studies (Hama *et al.*, 2009; Freedman *et al.*, 2007). Metabolites of the tobacco smoke are carcinogens, which can bind covalently to DNA and form bulky adducts. These DNA adducts can result in mutations that potentially could initiate or promote carcinogenesis. Adduct formation and persistence plays an important role in carcinogenesis, the balance between activation, detoxification, and DNA repair of these lesions is a significant factor in determining individual susceptibility to cancer (Sun *et al.*, 2007).

Like smoking, alcohol alone did not show prevalence in inducing mutations, combined with other habits especially chewing showed high frequency of mutations than with smoking alone. The other interesting thing was that chewing alone had high prevalence in inducing mutations than smoking and alcohol. The combination of two or more factors (alcohol with smoking and alcohol with chewing) were found to have increased frequency in causing mutations in the study. Chewing and alcohol habits were more prevalent in inducing mutations (Table 2). This could be the possible reason for the increased prevalence of female HNC cases with high frequency of mutations in our population. Many of them were habituated to chewing of tobacco components, so the frequency of alteration in the gene affected their exposure intensity (Tables 2 and 3). Surprisingly, the mutational frequency in non-exposure group was also significantly high in HNC disease. This indicates that the genetic variations of EGFR might play an important role in the occurrence of HNC disease. Earlier studies reported that gender, age (≤60 years), and smoking history were not associated with EGFR mutations (Na *et al.*, 2007). In HNC cases, the mutations were more in the age group of 45–65 years (Table 5).

The identification of the EGFR, TK domain mutations is an emerging area because genetic alterations in EGFR may affect the response to TKI. Anti-EGFR treatment can prevent activation of downstream signaling pathways such as the PI3K/Akt, RAS/Erk, and STAT pathways resulting in the inhibition of cellular proliferation and induction of apoptosis. Patients harboring mutations had a much higher response rate that is translated into improved survival times compared to wild type patients treated with EGFR, TKI at least in NSCLC (Linardou *et al.*, 2009). Earlier studies reported that the response rates to TKI observed in NSCLC was, as similar as the response rates in HNC (Cohen *et al.*, 2003). Mutations in exon 20 could be in-frame duplication and/or insertion, such as A767_V769dupASV or H773_V774insH (Shigematsu *et al.*, 2005). They could also be point mutations. Examples of reported exon 20 mutations in literature are V765M, S768I, H773R, and T790M (Takano *et al.*, 2005; Hsieh *et al.*, 2006). Only hand full of reports are available on HNSCC, Na *et al.* (2007) in Korean population have found 7.40% of exon 20 mutations, which include T790M, C775Y, S784Y, F795S, Y801H, G810D, G810S, and Y813C. Hama *et al.* (2009) in Japanese population found a low percentage (2.40%) and reported V765G, Ins770G. Schwentner *et al.* (2008) found G796S (1.57%), T725T (0.60%) in Australian population.

Q787Q of EGFR was observed to be prevalent and significant in HNC cases with tobacco and alcohol habits (65.11%). The habitual tobacco and alcohol consumption in our population elevated the frequency of polymorphisms in *EGFR* gene. In Taiwanese, OSCC patients Q787Q was more prevalent and did not show any significance with clinical, pathological, and recurrence rate (Hsieh *et al.*, 2011). Although Q787Q mutations exist in HNSCC, NSCLC, bile duct, and gall bladder carcinoma little evidence of clinical significance associated with Q787Q

Table 5: Age vs. mutations in cases and controls.

Age	HNC (n = 129)	HNC with SNP's (n = 97)	Controls (n = 150)	Controls with SNP's (n = 69)
25–35	19	12	47	35
36–45	17	15	21	13
46–55	32	23	31	8
56–65	45	34	41	10
66–75	13	10	9	3
76–85	3	3	1	–

mutation has been reported and EGFR mutations are not thought to be prognostic in HNSCC. Taguchi *et al*. (2008) noted that Q787Q mutation has a good correlation with response to gefitinib which was an *in vitro* (cell lines) study. Kaneko *et al*. (2010) reported a high frequency, with 33% Q787Q mutation (n¼ 19, n¼ 57) in esophageal cancer, and suggested that Q787Q mutations in pretreatment biopsy specimens may be a clinically useful biomarker for predicting prognosis in patients with esophageal cancer. Earlier it was reported that though it is silent mutations the heterozygous *EGFR* mutation (G/A genotype) group showed higher sensitivity (lower IC_{50} values) to gefitinib than the *EGFR* wt (G/G genotype) group did (Hsieh *et al.*, 2006). In population heterozygous G/A genotype in cases was 62.10% and homozygous mutant A/A was 13.17%. Q787Q may show a similar response to TKI as reported in the literature.

EGFR polymorphisms of V765M, S768I, R776H, G779S, T783A, L798H, K806E, and L814P reported earlier to the gefitinib treatment (Wu *et al.*, 2008; Takano *et al.*, 2005). Silent mutation at G779G found that though it was a synonymous mutation the genotypic variations might influence the response to TKI as seen with Q787Q. However, S784Y polymorphism did not show any response to TKI (Na *et al.*, 2007). Insertion/duplication/deletion S768_D770dupSVD was reported to be sensitive for gefitinib treatment. Mutations associated with resistance to EGFR, TKI are D761Y, T790M, and K806E (Wu *et al.,* 2008), studies on R776H and L798H might respond to the gefitinib treatment. It is therefore important to emphasize that the study of EGFR mutations in HNC to correlate the ethnic variations and habitual exposure to various tobacco forms and alcohol which play a major role in disease progression.

Conclusion

In conclusion the polymorphisms in EGFR were significant in HNC cases, the risk factors like tobacco and alcohol alone and in combination influenced the prevalence of SNPs. The observed SNPs in healthy volunteers indicated the importance of relative risk to HNC disease. These mutations were known to increase growth factor signaling cascade and confer susceptibility to the inhibitors. Hence, screening for mutations in the *EGFR* gene will be very useful as a biomarker for disease and therapy in HNC. Also a large body of experimental and clinical work supports the view that EGFR is a relevant target for cancer therapy.

Ethical Approval

This study was approved by the Institutional Ethics Committee of Mahavir Hospital and Research Centre, Hyderabad, Andhra Pradesh, India. Samples and biopsies were collected after informed consent from cases and controls, which formed the study group.

Conflict of Interests

None declared.

Authors' Contributions

All authors contributed equally to this study.

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