The Association Between Elevated EphB4 Expression, Smoking Status, and Advanced-Stage Disease in Patients With Head and Neck Squamous Cell Carcinoma

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Objective: To examine the expression of EphB4 in tumor tissue, surrounding normal tissue, and metastatic lymph node in patients with head and neck squamous cell carcinoma (HNSCC) and to evaluate its association with disease stage and smoking.

Design: A retrospective study.

Setting: University of Southern California–University Hospital, University of Southern California and Los Angeles County Medical Center, and Department of Otolaryngology–Head and Neck Surgery, University of Southern California, Los Angeles.

Patients: Forty-eight patients with different stages of HNSCC (I-IV) were enrolled into this study. Staging was based on the staging system of the American Joint Committee on Cancer.

Main Outcome Measures: EphB4 expression in tumor tissue, surrounding normal tissue, and metastatic lymph node was evaluated by immunohistochemical analysis, Western blot, and real-time polymerase chain reaction. EphB4 expression was then compared between patients based on disease stage and smoking status.

Results: EphB4 expression was detected in all tumor specimens and metastatic lymph nodes of patients with HNSCC, but expression levels were higher in the metastatic lymph nodes. There was a statistically significantly higher mean EphB4 protein expression and EphB4 gene amplification in patients with advanced disease (stage III or IV) vs patients with initial disease (stage I or II) and in smokers vs nonsmokers.

Conclusions: Overexpression of EphB4 is associated with advanced stages of HNSCC as well as with patients who smoke. These data are the first to demonstrate the association of EphB4 with advanced stages of disease and smoking in HNSCC and hence provide a strong rationale for targeting EphB4 for HNSCC therapies.

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HEAD AND NECK SQUAMOUS cell carcinoma (HNSCC) is the sixth most common cancer, with more than 900,000 cases diagnosed each year, and is one of the major causes of cancer death worldwide. In the United States alone, 50,000 new cases and 8000 deaths are reported each year. It affects the oral cavity, the oropharynx, the larynx, and the hypopharynx. The most important etiologic agents in HNSCC are tobacco and alcohol. A large-scale prospective study determined that the relative risk of death due to cancer among smokers vs nonsmokers older than 35 years was 27.5 for oral and pharyngeal cancer and 10.5 for laryngeal cancer.

Although surgery and radiotherapy are highly effective treatments for HNSCC early-stage disease (stage I or II), with cure rates ranging from 70% to 83%, advanced disease (stage III or IV) remains difficult to control, with an estimated 5-year survival rate of 30% to 40%. Therefore, prevention and early diagnosis of high-risk premalignant lesions are high priorities for reducing morbidity and mortality in head and neck cancer. In addition, regional metastasis is an important factor in the prognosis and choice of treatment for patients with HNSCC. The presence of nodal metastasis significantly affects the survival of the patient.

Angiogenesis plays an important role not only in tumor growth but also in tumor metastasis. Receptor tyrosine kinases (RTKs) have emerged as critical molecules in regulating many aspects of angiogenesis. Three families of RTKs have been implicated in angiogenesis: the VEGF family, the angiopoietin/Tie 2 family, and the ephrin/Eph family. The Eph receptor families are RTKs, transmembrane proteins that, on receiv-
ing an external stimulus, respond by transmitting a signal to the inside of the cell. The ephrins are the ligands of Eph receptors and are divided into an A subclass, which contains 8 members (EphA1-EphA8), and a B subclass, which contains 5 members (EphB1-EphB4 and EphB6). The EphA subclass is tethered to the cell membrane by glycosyl phosphatidylinositol, and the EphB subclass members have a transmembrane domain that is followed by a short cytoplasmic region.

Eph receptors and ephrins are involved in a number of biological functions including vascular development, tissue border formation, cell migration, axon guidance, and synaptic plasticity. Recently, several studies have shown high expression of the Eph family of RTKs in tumors. Aberrant expression of Eph receptors may be associated with increased potential for tumor growth, tumorigenicity, and metastasis. While there are limited data on the protein levels of EphB4 in cancers, there is an even greater deficit of data on the biological significance of this protein in tumor biology. EphB4 is a very early marker of venous endothelial cells, whereas its ligand, EphrinB2, reciprocally labels arterial endothelial cells. High expression of EphB4 has been reported in hematologic, breast, endometrial, and colon cancers. Evidence of a potential role of EphB4 as a tumor promoter comes from EphB4/neuT transgenic mice, which develop tumors more rapidly than neuT transgenic mice.

Our research group has previously reported expression of EphB4 in HNSCC primary tumor and cervical node metastasis. In the present report, we extend those findings by showing that EphB4 was expressed in 48 of 48 clinical HNSCC samples, and that the expression of EphB4 was higher in advanced disease (stages III and IV) vs initial disease (stages I and II). We further demonstrate that expression of EphB4 is higher in those patients who have a history of cigarette smoking. However, there is no relation with EphB4 and patient’s alcohol consumption status. Based on the data presented here and the role that EphB4 is known to play in angiogenesis, targeting EphB4 in head and neck tumors has the potential to directly inhibit tumor cell viability as well as block angiogenesis that indirectly supports tumor growth and metastasis.

**METHODS**

**PATIENT SELECTION**

A total of 48 patients with HNSCC underwent surgical resection at the University of Southern California-University Hospital and University of Southern California and Los Angeles County Medical Center and were prospectively entered into the study. The study was approved by the appropriate institutional review board. Demographic data were collected from the patient hospital records. The staff members who collected the demographic data had no knowledge of the status of EphB4 expression in the patient tumors. Perioperative data included the TNM stage of the head and neck cancer (stages I-IV according to the staging system of the American Joint Committee on Cancer), the site of the primary tumor, and the pathologic grade of the neoplasm on light microscopic examination. Tumor and normal adjacent tissues were collected in all 48 cases, while lymph nodes positive for tumor were harvested and analyzed in 17 cases. In patients with no clinical evidence of regional lymph node metastasis, no lymph node specimens were taken for analysis at the time of surgery. The tumor tissues were not laser dissected; therefore, to control for the amount of histologically normal tissue within the tumor sample, we used tumor tissue sections that showed more than 70% of tumors cells by hematoxylin-eosin staining. Oral mucosa from 10 healthy individuals with a history of smoking was also obtained. Each tissue specimen was given a unique identification number and sent to the laboratory for analysis. The laboratory was blinded to the status of the tissue specimen.

**REAGENTS**

Antibodies to EphB4 (C-16) and EphrinB2 (P20) were purchased from Santa Cruz Biotech (Santa Cruz, Calif). Additional monoclonal antibodies to the extracellular domain of EphB4 were generated in house (25D, 94D, and 265D).

**IMMUNOHISTOCHEMICAL ANALYSIS**

Five-micrometer sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and hydrated. Antigen epitope retrieval was performed by boiling slides in 10mM sodium citrate buffer (pH 8.5) at 80°C for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in phosphate-buffered saline for 10 minutes followed by blocking of nonspecific sites with SuperBlock blocking buffer (Pierce Biotechnology Inc, Rockford, Ill) for 1 hour, both at room temperature. Sections were incubated with primary antibody to EphB4 (C-16) overnight at 4°C. After washing in phosphate-buffered saline, antibody binding was localized with appropriate biotinylated secondary antibody and avidin/biotinylated horseradish-peroxidase complex (Vector Laboratories, Burlingame, Calif). Sections were then stained with DAB reagent (Vector Laboratories) and counterstained with hematoxylin-eosin. Routine negative controls included deletion of primary and secondary antibody and substitution of normal IgG isotope for primary antibody. The number of cells staining positive was counted by a blinded observer in 5 random high-power fields. The antibodies used in immunohistochemical analysis do not react with other members of the EphB and EphA families.

**WESTERN BLOT**

Cell lysates were prepared as previously described. Typically, 10-µg proteins from whole cell lysate were fractionated on a 4% to 20% Tris glycine polyacrylamide gel, electrotransferred to polyvinyl difluoride membrane, and probed with primary antibody overnight. Blot was stripped with Restore Western blot stripping buffer (Pierce Biotechnology Inc) and reprobed with β-actin to confirm equivalent loading and transfer of protein. Signal was detected using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc). Western blots were digitized, and the relevant protein bands of EphB4 and β-actin were quantified (Fluro-S Multi-Imager System; Bio-Rad Laboratories, Hercules, Calif). Western blots of tissue from primary tumor, lymph node metastases, and uninvolved tissue were carried out to determine the relative levels of EphB4 expression in these sites.

**REAL TIME PCR**

Real-time polymerase chain reaction (PCR) analysis was used to find EphB4 gene copy number in the DNA. Gene amplification was analyzed by quantitative PCR. The DNA was extracted from peripheral blood monocytes of healthy donor and
patient specimens, including normal, tumor, and lymph node specimens, using the Blood and Cell Culture DNA Midi Kit (Qiagen, Valencia, Calif) according to the manufacturer’s instructions. Quantitative PCR was performed on 50 ng of DNA on the Stratagene MX3000P system (Stratagene, La Jolla, Calif) using SYBR Green I Brilliant Mastermix (Stratagene) according to the manufacturer’s instructions with a thermal profile of 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, 64°C for 1 minute, and 72°C for 1 minute. The primers were EphB4 forward: 5′-TCC TGC AAG GAG ACC TTC AC-3′; EphB4 reverse: 5′-CAG AGG CCT CGC AAC TAC AT-3′; GAPDH forward: 5′-GAG GGG TGA TGT GGG GAG TA-3′; and GAPDH reverse: 5′-GAG CCT CCC GTT CAG CTC AG-3′. Amplification signal for EphB4 was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and gene copy number was normalized to the healthy donor peripheral blood monocytes.

STATISTICAL ANALYSIS

Analysis of variance was used to compare the difference in EphB4 expression between the tumor tissue, surrounding normal tissue, and lymph node. The t test was used to compare EphB4 expression within each demographic and clinicopathologic category. Linear regression analysis was performed to assess the predictive significance of the demographic and clinicopathologic factors for Ephb4 expression. For all purposes, a 2-sided P value <.05 was considered significant. All statistical analysis was performed using the SAS statistical package (version 8.2; SAS Institute, Cary, NC).

PATIENT CHARACTERISTICS

Forty-eight patients with HNSCC were enrolled in the study. The clinicopathologic features of the patients are listed in Table 1. Of the 48 patients in the study, 36 (75%) were male and 12 (25%) were female, with a mean age of 62 years (age range, 40-83 years). Most (63%) of the patients were white, followed by Hispanic (17%), Asian (15%), and African American (4%). Patients who did not belong to any of the above ethnic backgrounds were grouped under “other” (2%). Twenty (42%) of the 48 patients gave a history of alcohol intake. Thirty-eight (79%) of the 48 patients had advanced-stage disease (III or IV), which is what we expect to find in a tertiary care center. Twenty (42%) of the 48 patients had metastatic lymph nodes. Oral cavity (22/48; 46%) and oropharynx (13/48; 27%) were the most common sites involved in the study population, followed by neck (6/48; 13%), facial bone (5/48; 10%), larynx (1/48; 2%), and hypopharynx (1/48; 2%) (Table 1). Sixteen (33%) of the 48 patients had radiation therapy before surgery vs 7 (15%) of the 48 who had chemotherapy before surgery. Seventeen (35%) of the 48 patients had tumor that had metastasized to the lymph node of the neck region (Table 1).

EXPRESSION OF EphB4 IN HNSCC

We studied the expression of EphB4 in 48 human tumor tissues and 17 lymph nodes of patients with HNSCC by immunohistochemical analysis. The lymph nodes were from patients who had regional lymph node metastasis. EphB4 expression was observed in all cases of tumor tissue and metastatic lymph node with varying intensity of staining compared with normal tissue from the same patient. We noted a trend toward high intensity of staining in advanced-stage disease compared with initial-stage disease (Figure 1). No significant expression of EphB4 was observed in normal tissue from the same patient. The hematoxylin–eosin–stained tumor cells are Figure 1). We also evaluated EphB4 expression in the oral mucosa from healthy individuals with a history of smoking. None of these specimens showed expression of EphB4 (Figure 1D and E).

Western blot analysis was performed on 48 tumor tissue specimens, normal tissue specimens, and 17 metastatic lymph node samples. EphB4 expression was observed in each of the tumor samples. All tumor-positive

| Character | Finding |
|-----------|---------|
| Age, mean (range), y | 62 (40-83) |
| Sex | Male 36 (75)
Female 12 (25) |
| Ethnicity | White 30 (63)
Hispanic 8 (17)
Asian 7 (15)
African American 2 (4)
Other 1 (2) |
| Clinical stage† | IV 33 (69)
III 5 (10)
II 6 (13)
I 4 (8) |
| Radiation therapy before surgery | Yes 16 (33)
No 32 (67) |
| Chemotherapy before surgery | Yes 7 (15)
No 41 (85) |
| Family history of any cancer | Yes 11 (23)
No 37 (77) |
| Lymph node involvement | Yes 17 (35)
No 31 (65) |

*Unless otherwise indicated, data are reported as number (percentage) of study patients.
†Staged by the TNM staging system of the American Joint Committee on Cancer.
were quantitated (Fluro-S Multi-Imager System; Bio-Rad Laboratories, Hercules, Calif). The relevant protein bands of EphB4 (120 kDa) and β-actin (40 kDa) were quantitated (Fluro-S Multi-Imager System; Bio-Rad Laboratories, Hercules, Calif).

**Table 2. Findings From Western Blot Analysis of EphB4 Expression in Cancerous vs Normal Tissue Samples***

| Tissue                      | EphB4 Expression, Mean (SE) | P Value† |
|----------------------------|-----------------------------|----------|
| Normal tissue              | 1.0 (0.02)                  | <.001    |
| Tumor tissue               | 3.5 (0.29)                  | <.001    |
| Metastatic lymph nodes      | 5.2 (1.20)                  | <.001    |

*The relevant protein bands of EphB4 (120 kDa) and β-actin (40 kDa) were quantitated (Fluro-S Multi-Imager System; Bio-Rad Laboratories, Hercules, Calif).

†From analysis of variance.

lymph nodes showed EphB4 expression that was either equal to or greater than that of the primary tumor. Minimal or no EphB4 expression was observed in the adjacent normal tissue. There was a statistically significant difference in the mean EphB4 expression between the tumor tissue (3.49), tumor-positive lymph node (5.21), and normal tissue (1.02) (P < .001) (Table 2).

As detailed in **Table 3**, patients with advanced disease (stage III or IV) have a significantly higher mean (SE) expression of EphB4 than patients with initial disease (stage I or II) (3.8 [0.27] vs 2.1 [0.60]) (P = .01). EphB4 expression was higher in tumor tissues of patients with a smoking history than in nonsmokers (3.8 [0.52] vs 2.1 [0.37]) (P = .01). There was no significant difference in the mean (SE) EphB4 expression in tumor tissues of patients with a history of alcohol intake compared with that of patients with no history of alcohol intake (3.1 [0.40] vs 2.8 [0.50]) (P = .40). Also, there was no significant difference in the expression of EphB4 in tumor tissues based on lymph node involvement and radiation therapy before surgery (Table 3). In addition, EphB4 in metastatic lymph node samples of smokers showed a higher mean (SE) expression than in nonsmokers (7.7 [1.25] vs 4.2 [0.7]) (P = .02). There was no statistically significant difference in EphB4 expression within rest of the categories in metastatic lymph nodes and normal tissues (Table 3).

After stratifying the EphB4 expression based on smoking status, we noted a higher expression of EphB4 in patients with advanced disease and lymph node involvement than in those with initial disease and no lymph node involvement (Figure 2). These results were further confirmed by real-time PCR, which quantified the EphB4 gene amplification in tumor tissue and metastatic lymph nodes. There was a statistically significant difference in mean (SE) EphB4 gene amplification in patients with advanced disease (stage III or IV) vs those with initial disease (stage I or II) (2.5 [0.10] vs 0.9 [0.21]) (P < .001) and in smokers vs nonsmokers (2.1 [0.18] vs 1.4 [0.13]) (P = .01) (Table 4). There was no significant difference in the EphB4 gene amplification in tumor tissues based on alcohol consumption (P = .50), lymph node involvement (P = .80), or radiation therapy before surgery (P = .10) (Table 4). As listed in Table 4, there was no significant difference in the EphB4 gene amplification found in metastatic lymph nodes based on smoking, alcohol consumption, or lymph node involvement status (P > .05 for each).

**COMMENT**

Receptor protein tyrosine kinases transmit extracellular signals inside the cell and play a critical role in cell growth, differentiation, and migration. EphB4 is an RTK that along with its ligand, EphrinB2, is essential for commitment to venous or arterial phenotype. EphB4 and EphrinB2 are also required for vessel maturation and remodeling. Studies have shown that EphB4- and EphrinB2-targeted gene knockouts in mice show similar phenotypes, thus demonstrating their concomitant role in angiogenesis. Several studies have recently shown that high expression of Eph/ephrin may be associated with a greater susceptibility to tumor growth, tumorigenicity, and metastasis.9,11

In the present study, we examined the expression of EphB4 in different tissues and at different stages of HNSCC development, examining its potential role in tumor growth and metastasis. There was a higher expression of EphB4 in tumor and lymph nodes than in normal tissue of the same patient, with EphB4 being overexpressed in all patients with HNSCC regardless of the stage. However, EphB4 expression was higher in stage III and IV disease than in stage I and II. Furthermore, EphB4 expression was found to be higher in the metastatic lymph node than in the primary tumor. Since EphB4 is involved in vessel maturation and neovascularization, it may also play a crucial role in tumor growth and metastasis. EphB4 activation has also been shown to cause increased proliferation and survival of human microvascular endothelial cells via increased PI3K activity and phosphorylation of AKT and MAPK.18 The metastatic potential of EphB4 in cell migration and invasion is associated with EphB4 induction of MMP2 and MMP9.18 Thus EphB4 overexpression confers increased cell survival and invasion potential in cells where EphB4 is normally expressed. Recently, microarray analysis of hypopharyngeal carcinoma showed EphB4 among the 6 overexpressed genes associated with tumorigenesis.19
The molecular mechanism by which EphB4 induces tumor growth has been well established. Increased EphB4 protein expression has been reported in breast carcinoma, endometrial carcinoma, and colon carcinoma.9,11,20 Recently, our group has shown higher expressions of EphB4 in prostate carcinoma and mesothelioma.14,15 EphB4 expression is induced by epidermal growth factor/epidermal growth factor receptor (EGF/EGFR) and insulinlike growth factor-I/insulin-like growth factor-IR; it is also induced by loss of PTEN and p53. Knockdown of the EphB4 protein using EphB4 short interfering RNA or antisense oligodeoxynucleotide significantly inhibits cell growth and viability, migration, and invasion, and induces apoptosis in prostate carcinoma and mesothelioma cell lines.19,15 Several investigators have shown that mutations in specific genes (p53, p16, and Rb) and alteration of their expression (EGFR, Cyclin D, and ras) lead to the development of HNSCC.8,10,21-23 Similar studies on EGFR gene amplification have demonstrated an increase in the MAPK signaling pathways, thus implicating EphB4 in cell proliferation.24

The development of HNSCC is known to be strongly associated with tobacco use. More recently, many of the molecular mechanisms of tobacco-induced carcinogenesis have been discovered, particularly for lung cancer. However, little is known with regard to the mechanisms for HNSCC. Rodriguez et al24 showed that tobacco use alone led to a 20-fold increased risk of HNSCC and thus plays a major role in HNSCC development.24 Tobacco has been linked with altered metabolism, genomic instability, and interaction with the extracellular environment in HNSCC. Recently, it was shown that many of the genes and growth factors associated with HNSCC are activated or mutated directly by tobacco-related substances.25,26

### Table 3. Findings From Western Blot Analysis of EphB4 Expression by Patient Characteristic in Different Tissue Samples*

| Characteristic                  | Tumor (n = 48) | P Value† | Metastatic Regional Lymph Node (n = 17) | P Value† | Surrounding Normal Tissue (n = 48) | P Value† |
|--------------------------------|---------------|----------|----------------------------------------|----------|-----------------------------------|----------|
| Disease stage                  |               |          |                                        |          |                                   |          |
| Initial (I or II)              | 2.1 (0.60)    | .01      |                                        |          |                                   | .20      |
| Advanced III or IV             | 3.8 (0.27)    |          |                                        |          |                                   |          |
| Smoking                        |               |          |                                        |          |                                   |          |
| Yes                            | 3.8 (0.52)    | .01      | 7.7 (1.25)                             | .02      | 0.9 (0.06)                        | .20      |
| No                             | 2.1 (0.37)    |          | 4.2 (0.70)                             |          |                                   |          |
| Alcohol consumption            |               |          |                                        |          |                                   |          |
| Yes                            | 3.1 (0.40)    | .40      | 6.7 (0.82)                             | .20      | 0.9 (0.04)                        | .10      |
| No                             | 2.8 (0.50)    |          | 5.1 (1.25)                             |          |                                   |          |
| Lymph node involvement         |               |          |                                        |          |                                   | .40      |
| Yes                            | 3.1 (0.55)    | .40      |                                        |          |                                   |          |
| No                             | 2.8 (0.31)    |          |                                        |          |                                   |          |
| Irradiation before surgery     |               |          |                                        |          |                                   |          |
| Yes                            | 2.9 (0.51)    | .90      | 7.1 (1.11)                             | .10      | 0.9 (0.06)                        | .50      |
| No                             | 3.0 (0.33)    |          | 4.8 (0.92)                             |          |                                   |          |

Abbreviation: NA, not applicable (there was no comparison group for analysis because lymph nodes of patients with no regional lymph node metastasis were not analyzed).

*Unless otherwise indicated, data are reported as mean (SE) expression values adjusted for the applicable characteristic.
†From independent t test comparing levels of EphB4 expression within each category of the applicable characteristic.

### Figure 2. EphB4 expression in nonsmokers (A) and smokers (B) with head and neck squamous cell carcinoma. EphB4 expression was normalized with β-actin. LN indicates metastatic regional lymph nodes. Symbols indicate individuals.
In the present study, we have shown that smokers with HNSCC had a higher expression of EphB4 than nonsmokers with HNSCC. Tobacco metabolites or tobacco-induced epigenetic changes may activate EphB4, thus initiating the angiogenesis cascades necessary for tumor growth. Based on the recent evidence directly linking smoking with molecular alterations, it is not surprising to see that tobacco usage corresponds with increased levels of EphB4. However, the direct interaction between tobacco usage and EphB4-induced HNSCC remains to be studied. Furthermore, the nonassociation between alcohol consumption and EphB4 levels may relate to the different mechanisms of toxin-induced carcinogenesis. Thus it is the combination of genetics and environmental factors that are associated with HNSCC.

Based on our results, EphB4 may represent a new target for HNSCC therapy. Tyrosine kinases are commonly overexpressed or aberrantly expressed in human cancers. Drugs that inhibit such tyrosine kinases are among the first success stories in targeted therapeutics for cancer. Thus, the identification of novel targets (such as EphB4), an understanding of how overexpression or activation of multiple targets might interact to affect responses, and the testing of combination therapies will likely have a significant impact on the treatment of cancers.

Head and neck squamous cell carcinoma represents a devastating disease that degrades quality of life due to both tumor and treatment morbidity. By studying the various molecular mechanisms by which HNSCC metastasizes and affects survival, we hope to elicit new prognostic and screening markers to help dictate novel treatment methods and modify current treatment protocols to correspond with the aggressiveness or resistance to treatment of the tumor, based on its molecular makeup. Current studies have implicated various genes as potential biomarkers. EphB4 may represent such a prognostic marker in HNSCC diagnosis, based on the primary data presented here. The use of molecular markers will allow better identification of premalignant tumors and early lymph node metastasis and guide surgical procedures for cleaner margins and better use of postoperative adjuvant therapy. Using these markers, it is our hope to reduce the morbidity and mortality associated with HNSCC, ultimately improving survival and quality of life in patients with HNSCC.

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Author Contributions: Dr Sinha had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Sinha, Masood, and Gill. Acquisition of data: Masood. Analysis and interpretation of data: Sinha and Masood. Drafting of the manuscript: Mazhar, Chinn, and Dhillon. Critical revision of the manuscript for important intellectual content: Sinha and Masood. Statistical analysis: Mazhar. Obtained funding: Sinha and Rice. Administrative, technical, and material support: Sinha. Study supervision: Sinha and Masood.

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