

Increased Levels of Circulating and Tissue mRNAs of Oct-4, Sox-2, Bmi-1 and Nanog in ESCC Patients: Potential Tool for Minimally Invasive Cancer Diagnosis

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Background: Early stages of esophageal cancer lack a specific symptom, a reliable biomarker and accurate non-invasive diagnostic modalities prompting the pressing need for identification of a marker for early diagnosis of this disease.

Methods: In the present study we investigated the levels of circulating and tissue mRNAs of Oct-3/4, Sox-2, Nanog and Bmi-1 in esophageal cancer patients using Reverse-Transcription Polymerase Chain Reaction (RT-PCR) with the aim of evaluating their potential as minimally invasive diagnostic markers.

Result: Increased transcript levels of Oct-4, Sox-2, Bmi-1 and Nanog were detected in (92%), (95%), (75%) and (67%) of the esophageal cancer tissues, respectively as compared with the matched distant normals.

Conclusion: Interestingly, most of the preneoplastic tissues exhibited increased transcript levels of these stemness markers suggesting their role in early stages of esophageal tumorigenesis. Furthermore, the detection of elevated levels of circulating mRNAs of Oct-4 and Nanog in sera of esophageal cancer patients emphasizes their potential as minimally invasive diagnostic markers for esophageal cancer.

Keywords: Oct3/4, Sox-2, Bmi-1, Nanog, pluripotent

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Introduction

Human esophageal cancer occurs worldwide with a variable geographic distribution and ranks eighth in order of occurrence and sixth as the leading cause of cancer mortality, affecting men more than women.¹ At diagnosis, nearly 50% of cases have cancer that extends beyond the primary locoregional confines and 75% of patients requiring surgery have proximal lymph node metastasis. At this stage, therapeutic modalities are limited in their success. Detecting cancers in early stages even in the premalignant state, means that current or future treatment modalities might have a higher likelihood of a true cure. In spite of the use of modern surgical techniques combined with various adjuvant treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate of esophageal cancer patients still remains less than 10%. Hence, early detection of esophageal cancer lacks a specific symptom, a specific biomarker and accurate and reliable diagnostic, non-invasive modalities. Traditional methods of treating cancer include use of invasive or mildly invasive diagnostic tests, biopsy, and histological examination and so on. The invasive, unpleasant and inconvenient nature of the current diagnostic procedures limits the application of till date proven tumor markers. Hence, there is a pressing need for establishment of novel non-invasive biomarkers for early tumor diagnosis and monitoring response to therapy and prognosis.

Stem cell related genes, Oct 3/4, Sox2, Nanog and Bmi-1 are the factors responsible for the maintenance of the proliferation and pluripotent character in the Embryonic Stem Cells. Cancer cells, especially in poorly differentiated or undifferentiated tumors, have been characterized by many phenotypic traits similar to undifferentiated embryonic cells, indicating that stem cell related genes *viz.* Oct3/4, Sox2, Nanog and Bmi-1 may be expressed in solid tumors.

Oct-4, belongs to the POU (Pit-Oct-Unc) transcription factor family.² The POU family of transcription factors can activate the expression of their target genes through binding an octameric sequence motif of an AGTCAAAT which is a consensus sequence.^{3a} This gene encodes a transcription factor containing a POU homeodomain. Oct-4 plays a key role in the maintenance of proliferation potential of embryonic stem cells as well as the human adult stem cells.⁴ Moreover, Pesce and Scholar, 1990 have shown Oct-4

to be downregulated in all the differentiated somatic cell types *in vitro* and *in vivo*.^{3b}

Sox (SRY box) genes have been identified through their homology to the high mobility group (HMG) box (79 amino acids) of sex-determining factor SRY. The Sox2, a member of the Sox gene family which is an intronless gene encodes a group of transcription factors that are characterized by a highly conserved high-mobility group (HMG) domain.⁵ These genes are found throughout the animal kingdom, are expressed in a restricted spatial-temporal pattern, and play critical roles in stem cell biology, organogenesis, and animal development.^{6,7} For example, overexpression of Sox2 in mouse neural stem cells blocks their differentiation. Recently, SOX transcription factors have been found to be associated with human cancers.⁸

Nanog is another transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells. In humans, this protein plays a critical role in regulating the cell fate of the pluripotent inner cell mass during embryonic development.⁹ *In vitro*, Nanog mRNA is enriched in pluripotent cell lines such as embryonic stem, embryonic germ, and embryonic carcinoma cells but not in adult tissues. On differentiation of these pluripotent cells, Nanog expression is down-regulated. Takahashi et al 2007 reported that Nanog, works in concert with Oct-4 and Sox-2 to maintain pluripotency.¹⁰

Bmi-1 polycomb ring finger oncogene, also known as Bmi-1, is a protein which in humans is encoded by the BMI1 gene.¹¹ The mRNA transcribed from Bmi-1 gene is linear and 3251 bp in length and Bmi1 has a RING finger at the N-terminus and a central helix-turn-helix domain.¹² Bmi-1 has been reported to regulate p16 and p19, the cell cycle inhibitor genes and hence act as the oncogene rendering abnormal proliferative ability. Bmi-1 is necessary for efficient self-renewing cell divisions of adult hematopoietic stem cells as well as adult peripheral and central nervous system neural stem cells and is a downstream target in the Hedgehog (Hh) pathway.

Previous studies have demonstrated that many cancers express these genes and that their expression appears to be important for cancer cell survival. The Oct3/4 and Sox2 genes have also been shown to be expressed in human tumors including pancreatic, breast, esophageal and gastric carcinomas.¹³ Recent studies have shown the overexpression of Bmi-1 in



many somatic solid tumors such as colon carcinoma,¹⁴ non small lung cancer,¹⁵ breast cancer,¹⁶ head and neck cancer¹⁷ and gastric carcinoma.¹⁸ He et al 2009 have shown its overexpression in ESCC.¹⁹ Although NANOG is generally found to be expressed in germ cell tumors²⁰ as well as in somatic tumors like breast^{21,22} cervix,²³ oral cavity,²⁴ kidney,²⁵ and ovary,²⁶ however, there are reports of its expression in esophageal cancer. Recently, microarray analysis of “Tip”-Side Population cells of esophageal cancer cell lines showing stem cell like characteristics revealed differential expression of several important stem cell-related genes *viz.* Oct-4, Sox-2, Bmi-1, and ZFX.²⁷ Therefore, it might be expected that cancer cells will express genes in common with the very early embryonic cells, especially genes specifically associated with deprogramming, return to the undifferentiated and proliferative stem cell state, and the maintenance of that state. Moreover, the identification of circulating levels of these genes in sera of ESCC patients may be instrumental in development of these makers as a diagnostic tool for the early diagnosis of esophageal cancer. Thus, keeping in view the above mentioned facts the aim of the present study is to analyze the expression of stem cell markers *viz.* Oct-4, Sox-2, Bmi-1 and Nanog in tumor and sera in the ESCCs and evaluate their potential as minimally invasive blood based markers. To the best of our knowledge, this is the first study analyzing the levels of circulating mRNAs of these genes in sera of esophageal squamous cell carcinoma patients. It is also the first report showing the expression of Nanog in ESCCs.

Materials and Methods

Patients and clinicopathological data

collection, tissue and blood specimens

Tumor and matched distant nonmalignant esophageal tissue biopsy specimens from a distal site were collected from 33 esophageal diagnostic biopsies of patients who underwent endoscopy in Department of Gastroenterology, All India Institute of Medical sciences, New Delhi, India with the prior consent of patients. This study was approved by the institutional human ethics committee prior to its commencement. The sample collection was carried out for a period of 8 months from October 2009 to June 2010. The samples were collected and immediately snap frozen in liquid N₂ and stored at -80 °C till further use.

The clinical and pathological data were recorded in a predesigned Performa including age, gender, and alcohol consumption, tobacco and histological differentiation. The tumors were histopathologically graded as preneoplastic (8) and neoplastic (25). Of the 33 endoscopic biopsies collected 25 showed evidence of ESCC of different grade, 5 were hyperplastic and 3 showed evidence of dysplasia. Blood samples were collected from 17 out of 33 ESCC patients enrolled in the study whose tumors were analysed for the expression of stem cell related genes. Blood samples were also collected from 8 normal subjects. The sera was separated and stored at -80 °C till further use.

The esophageal squamous cell carcinoma (ESCC) cell line TE13 was used as positive control for gene expression studies. The cell line was developed from squamous cell carcinoma of esophageal origin²⁸ and was a kind gift from Dr. Pierre Haiuant, IARC, France. TE13 cells were cultured in a 1:1 mixture of Rose Park Memorial Institute (RPMI) supplemented with 10% FBS. Cells were grown in monolayer cultures in a humidified incubator (5% carbon-dioxide, 95% air) at 37 °C.

RNA Isolation from tissue specimens

Total RNA was isolated from the tissue samples using the RNA easy minikit (Qiagen, Denmark). Briefly, the lysate were prepared using the lysis buffer followed by the addition of ethanol to provide ideal binding conditions. The lysate was then loaded onto the RNAeasy Mini spin column. RNA binds and all contaminants were efficiently washed away in the subsequent washing steps. Pure, concentrated RNA was eluted in RNase free water.

RNA Isolation from sera samples

Total RNA was isolated from the sera samples using QIAamp Viral RNA minikit (Qiagen, Denmark) following the manufacturer's protocol. Briefly, the samples were first lysed under highly denaturing conditions to inactivate RNAses and to ensure the isolation of intact circulating RNA. Buffering conditions were then adjusted to provide optimum binding of the RNA to QIAamp membrane and then sample were loaded on the QIAamp spin column. The RNA bound to the membrane and the contaminants were washed away using two different buffers. RNA was eluted in RNAase free water.



The RNA isolated from both tissue and sera samples were quantified by spectrophotometry, absorbance taken at 260 nm. The RNA obtained from tissue and sera samples were in the range of (1–2 µg/µL) and (0.5–1 µg/µL), respectively.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The first strand cDNA was reverse transcribed from DNA free total RNA obtained from esophageal tissues and sera using random hexamers (Fermentas, Canada) and MMLV reverse transcriptase (Fermentas, Canada).

The cDNA thus obtained was PCR amplified using the gene specific primer sets for Oct-4, Sox-2, Bmi-1 and Nanog (Table 1). β -Actin was used as a control to optimize the amounts of cDNA generated from each sample. PCR amplification was carried out in a total volume of 20 µL containing 3 µL reverse transcribed cDNA, 1x PCR buffer, 0.3 mM dNTPs, 0.5 µM each primer, and 1 unit of Taq polymerase (Fermentas, Canada). After 5 min of initial denaturation, 35 amplification cycles of 1 min at 94 °C, 1 min at specific annealing temperature (Table 1), and 1 min. at 72 °C were carried out followed by a 10 min elongation at 72 °C. The PCR products were then electrophoresed on 1% agarose gel and the bands of specific sizes were observed.

Densitometric analysis was carried out using Image J software to compare the expression of these mRNAs in ESCCs and the matched distant non malignant esophageal tissue biopsies. For individual samples, the integrated intensity value of each band (sum of all the pixel intensity values in a given band) was

determined and the background was subtracted. Normalization was achieved by dividing the corrected integrated density value of the gene in each sample by corrected integrated density value of beta-actin gene in the corresponding sample. The ratio was considered as arbitrary units. The expression levels of Oct-4, Sox-2, Bmi-1 and Nanog in each tissue is represented by a histogram.

Real time RT-PCR

Real-time RT-PCR was performed with OPTICON 2 Real time PCR system (BioRad) in a total volume 25 µL containing 0.5 µM of each primer, 12.5 µL SYBR® *Premix Ex Taq* SYBR green mix (Takara, Japan) and 2.5 µL of cDNA. PCR reactions were prepared and heated to 95 °C for 1 minutes followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at specific T_m for 1 min, and extension at 72 °C for 1 minute. Fluorescence detection was performed at the end of each extension step. The cycle threshold (Ct) was recorded for each sample and 5 s RNA was used as the endogenous control for data normalization. Relative expression was calculated using comparative CT method. The quantitative real time RT-PCR amplification products were analyzed by melting curve analysis.

Statistical analysis

Statistical analyses of the data were performed using SPSS software version 13.0 (SPSS, Chicago). The associations between the Oct-4, Sox-2, Nanog and Bmi-1 and the clinicopathological parameters of the ESCC patients were examined using the Chi-Square test. A *P*-value of 0.05 has been defined

Table 1. Parameters for RT-PCR analysis of stem cell related genes.

Gene	Annealing temperature (°C)	Primer combination	Product length (bp)
Oct-4	58 °C	5' GAG GAG TCC CAG GAC ATC AA 3' 5' ACA CTC GGA CCA CAT CCT TC 3'	429 bp
Sox-2	64 °C	5' GCC TGG GCG CCG AGT GGA 3' 5' GGG CGA GCC GTT CAT GTA GGT CTG 3'	443 bp
Nanog	58 °C	5' GGGCGAGCCGTTTCATGTAGGTCTG 3' 5' ATTTTCATTCGCTGGTTCTGG 3'	438 bp
Bmi-1	57 °C	5' CAG CAA TGA CTG TGA TGC ACT 3' 5' GCC CAA TGC TTA TGT CCA CT 3'	799 bp
β actin	62 °C	5'-CAG CCA TGT ACG TTG CTA TCC AG-3' 5'-GTT TCG TGG ATG CCA CAG GAC-3'	646 bp



as the criterion for the statistical significance. All the *P*-values reported were 2-tailed.

Results

The expression of stem cell related genes Oct-4, Sox-2, Bmi-1, Nanog was analyzed in ESCCs (25), preneoplastic esophageal tissues (8) and matched distant non malignant tissues of the esophageal biopsy. As, there is heterogeneity in the origin of cells present in the endoscopic biopsy specimen, the expression of Oct-4, Sox-2, Bmi-1 and Nanog was analysed in the esophageal cancer cell line (TE13) as well. Stem cell related genes analysed in this study were expressed in the TE13 cell line indicating their expression occurs in the tumor cells (data not shown).

Elevated levels of Oct-4 mRNA were observed 23/25 (92%) in of the ESCC tissues (Table 2). Interestingly, 5/6 (83.3%) hyperplastic tissues showed overexpression of Oct-4 mRNA as compared to distant non malignant tissues which show low or no detectable expression suggesting its role in early stages of esophageal tumorigenesis. One of the two dysplastic tissues showed expression of Oct-4. Figure 1A shows the elevated transcript levels of Oct-4 in representative ESCCs and matched distant nonmalignant esophageal tissues with reference to the house keeping gene β -actin shown in (Fig. 1G). Densitometric analysis showed evident increase in its expression in preneoplastic and neoplastic cancerous tissues as compared to the matched non malignant esophageal tissues (Fig. 2A). Moreover, 12/17 (70%) of the ESCC patients' sera samples show expression of Oct-4 mRNA as compared to no detectable expression in sera of normal subjects (Fig. 1B).

No significant correlation was observed between Oct-4 gene and clinicopathological parameters. All the sera specimens showing overexpression of Oct-4 mRNA also showed tissue positivity for its gene expression further corroborating the fact that presence of Oct-4 mRNA in patients sera is from the tumor tissue.

Of the 25 ESCCs, Sox-2, Nanog and Bmi-1 expression could be analysed in 22, 21 and 20 samples respectively due to less quantity of the rest of the samples.

Increased transcript levels of Sox-2 were observed in 21/22 (95%) of the ESCC tissues (Table 2). Of the five hyperplastic tissues analysed 4 (80%) tissues showed the overexpression of the Sox-2 mRNA as compared to non malignant tissue which showed no or low detectable expression hence it could be involved in the early phase of tumorigenesis with reference to Housekeeping gene β -actin shown in (Fig. 1G). Figure 1C shows the elevated transcript levels of Sox-2 in representative ESCCs and matched distant nonmalignant esophageal tissues. Densitometric analysis showed evident increase in its expression in preneoplastic and neoplastic cancerous tissues as compared to the matched non malignant esophageal tissues (Fig. 2B). Sox-2 did not show significant correlation with any of the clinicopathological parameters.

Overexpression of Nanog was observed in 14/21 (67%) of the ESCC tissues (Table 2). Figure 1E shows the elevated transcript levels of Nanog in representative ESCCs and matched distant nonmalignant esophageal tissues. Densitometric analysis showed evident increase in its expression in hyperplastic and neoplastic cancerous tissues as compared to the matched non malignant esophageal tissues (Fig. 2C). Interestingly, 4/5 (80%) hyperplastic

Table 2. Overexpression of Stem cell related mRNA transcripts in human ESCCs, preneoplastic and normal esophageal epithelium.

Tissue type	Oct-4 positive <i>n</i> (%)	Sox-2 positive <i>n</i> (%)	Nanog positive <i>n</i> (%)	Bmi-1 positive <i>n</i> (%)
ESCC	23/25 (92)	21/22 (95)	14/21 (67)	15/20 (75)
Preneoplasia (Hyperplasia and Dysplasia)	5/8 (63)	4/5 (80)	4/5 (80)	2/3 (67)
Age				
≤40	26/29 (90)	22/24 (92)	15/23 (65)	15/21 (71)
>40	3/4 (75)	2/3 (67)	3/3 (100)	2/2 (100)
Gender				
Male	19/22 (86.3)	15/18 (83.3)	12/17 (70.5)	10/15 (66.6)
Female	11/11 (100)	9/9 (100)	6/9 (67)	7/8 (88)

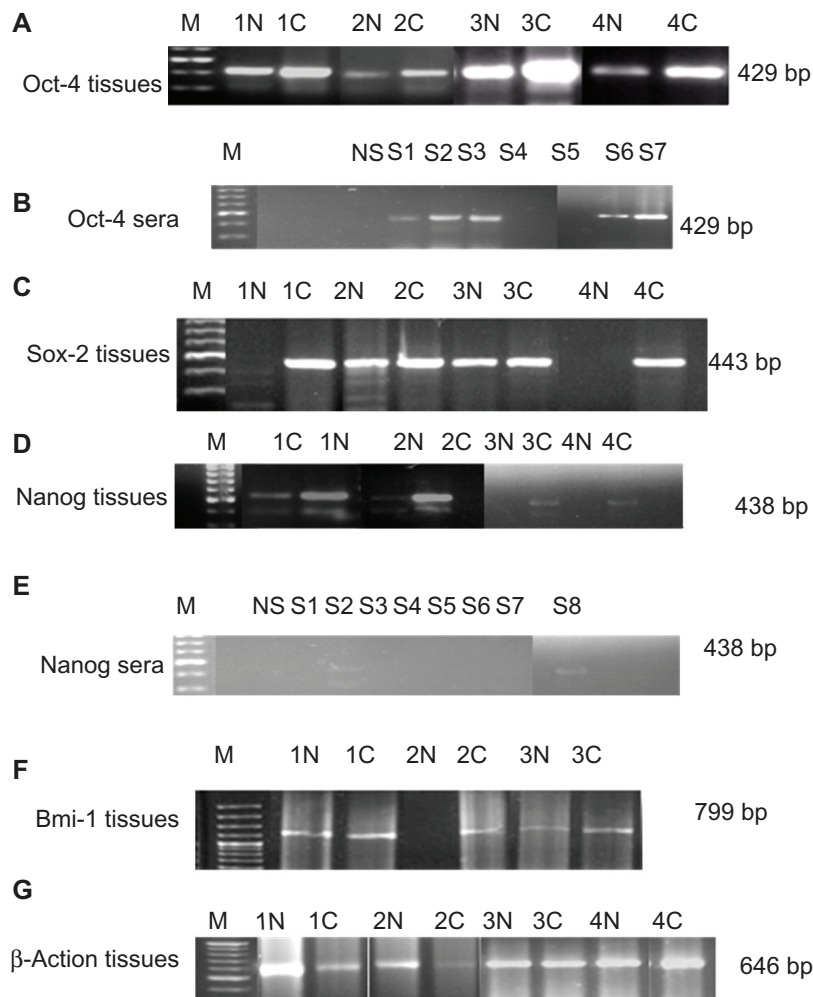


Figure 1. Representative 1% agarose showing the RT-PCR analysis of stem cell related genes in representative esophageal tissues. (A) shows a 429 bp Oct-4 amplicon in 4 distant normal tissues (N1-N4) and 4 ESCC tissues (C1-C4). (B) shows a 429 bp Oct-4 amplicon in 7 ESCC patients' sera (S1-S7) and 1 normal subject sera (NS). (C) shows a 438 bp Sox-2 amplicon in 4 distant normal tissues (N1-N4) and 4 ESCC tissues (C1-C4). (D) shows a 443 bp Nanog amplicon in 4 distant normal tissues (N1-N4) and 4 ESCC patients (C1-C4). (E) shows a 443 bp Nanog amplicon in 8 ESCC patients' sera (S1-S8) and 1 normal subject sera (NS). (F) shows a 799 bp Bmi-1 amplicon in 3 distant normal tissues (N1-N3) and 4 ESCC patients (C1-C3). (G) shows a 646 bp amplification of β -actin in the respective esophageal tissue samples.

patients showed the overexpression of the Nanog mRNA transcripts as compared to the distant non malignant tissues which showed no or low detectable expression level which could be the harbinger of this gene involved in early stages of tumorigenesis with reference to the House keeping gene β -actin shown in (Fig. 1G). Moreover, 10/17 (60%) of the ESCC sera samples shows expression of Nanog mRNA as compared to no detectable expression in sera of normal subjects (Fig. 1F). No significant correlation was observed between the Nanog expression and any of the clinicopathological parameter.

Elevated expression levels of Bmi-1 was found in the 15/20 (75%) of the tissues (Table 2, Fig. 1F). Densitometric analysis showed evident increase in its

expression in preneoplastic and neoplastic cancerous tissues as compared to the matched non malignant esophageal tissues (Fig. 2E). 2/3 (67%) of the Bmi-1 preneoplastic lesions showed increased Bmi-1 expression. There was no significant correlation between Bmi-1 expression the clinicopathological parameter.

Quantitative real time PCR performed in representative esophageal samples further validated the increased expression of stem cell related genes *viz.* Oct-4, Sox-2, Bmi-1 and Nanog in ESCCs as compared to matched non malignant esophageal tissues using comparative CT method (Fig. 3).

We also analyzed the correlation between the expressions of stem cell related genes analyzed. A statistically significant positive statistical correlation

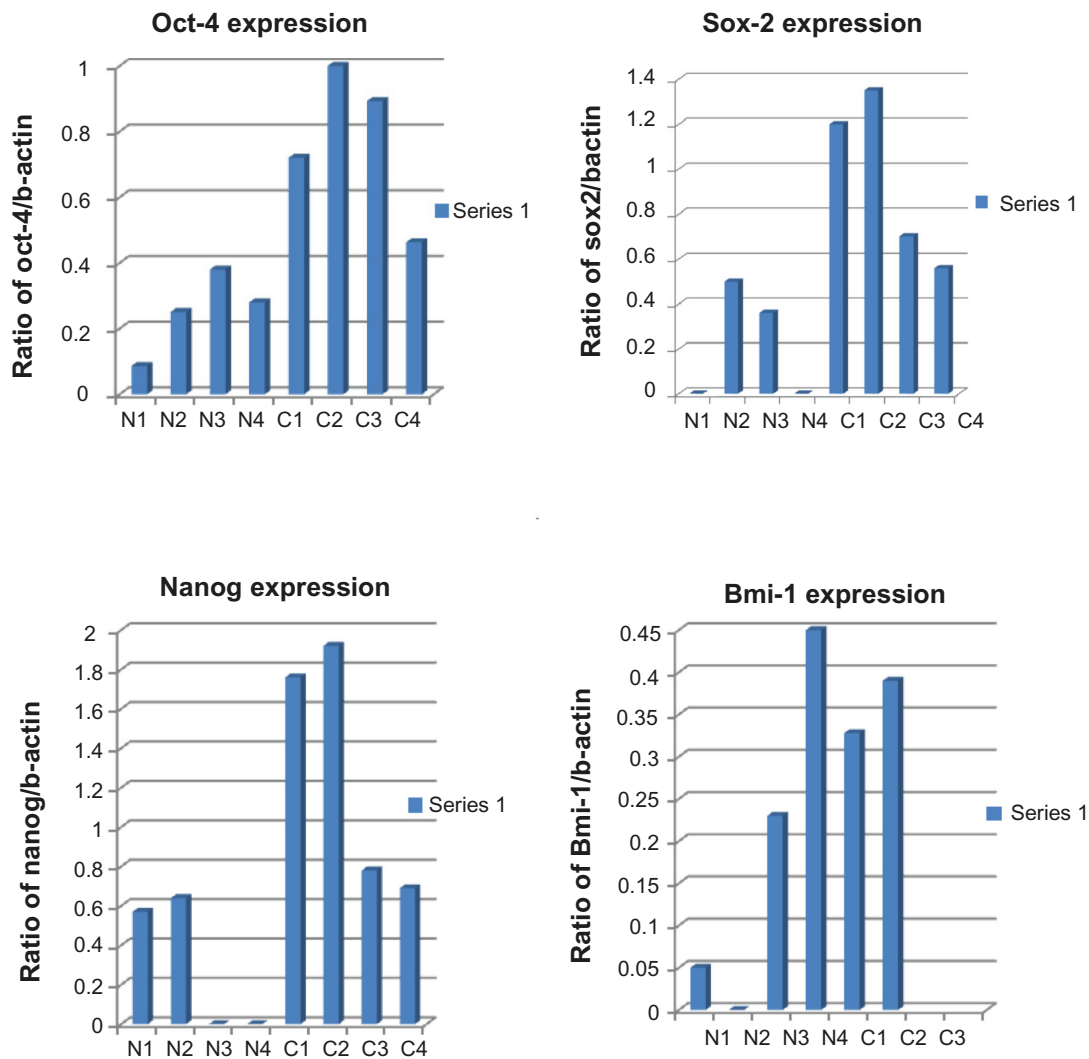


Figure 2. Histogram showing the expression levels of stem cell related genes relative to β -actin expression in representative esophageal tissues using densitometric analysis of RT-PCR products (A) shows the relative expression of Oct-4 in 4 distant normals (N1-N4) and 4 ESCC tissues (C1-C4). (B) shows the relative expression of Sox-2 in 4 distant normals (N1-N4) and 4 ESCC tissues (C1-C4) (C) shows the relative expression of Nanog in 4 distant normals (N1-N4) and 4 ESCC tissues (C1-C4) (D) shows the relative expression of Bmi-1 in 3 distant normals (N1-N3) and 3 ESCC tissues (C1-C3).

was observed between the expression of Oct-4 and Sox-2 ($P = 0.009$) indicating that their coexpression may play an important role in development of esophageal tumorigenesis.

Discussion

The self renewal and pluripotency are the hallmarks of the embryonic stem cells and it has been observed that deregulation of these pathways involved in differentiation and maintenance of proliferation. Oct-4, Sox-2 and Nanog render the reprogramming capability of adult cells into induced pluripotent cells.²⁹⁻³¹ Recently, the expression of the genes Oct-4, Sox-2 and Nanog has been reported in various cancers suggesting the tumorigenic potential of these genes.

Bmi-1, the first PcG protein found, is a chromatin modifier implicated in the tumorigenesis through negatively regulating the gene expression such as the INK4 A locus, which is thought to regulate p53 and the Rb signaling pathway in cooperation with c-myc.³²⁻³⁵

The present investigation on esophageal squamous cell carcinomas revealed several interesting observations. Firstly, it was observed that 92%, 95%, 67%, 75% of ESCC tissues showed increased expression of Oct-4, Sox-2, Nanog and Bmi-1 respectively. Secondly, most of the preneoplastic tissues were observed to be positive for the expression of these genes suggesting their role in early stages of esophageal tumorigenesis. Thirdly, a significant positive correlation

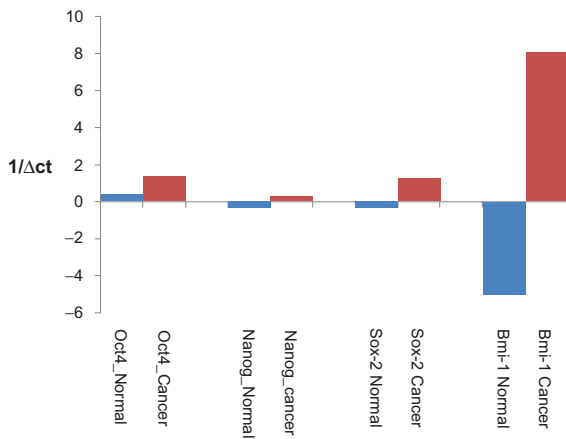


Figure 3. Histogram showing the expression levels of stem cell related genes Oct-4, Sox-2, Nanog and Bmi-1 expression in representative esophageal tissues using quantitative real time PCR. $\Delta ct = ct_{\text{target gene}} - ct_{\text{reference gene}}$

was observed between the expression of Oct-4 and Sox-2 genes (** $P = 0.009$). Fourthly, to the best of our knowledge this is the first study showing the elevated circulating levels of the mRNAs for these genes in the sera of the patients showing evidence of hyperplasia, dysplasia and ESCC indicating the possible potential of these markers to be used in noninvasive blood based diagnosis of the disease.

Oct3/4 expression has been studied earlier also in the case of many cancers and has shown significant expression in many other cancer types like the human breast cancer,³⁶ liver, pancreas, kidneys, mesenchyme, gastric stem cells,³⁷ HeLa³⁷ and MCF-7³⁸ cancer cell lines. Oct-4 has also been shown to be highly expressed in the bladder cancer.³⁹ Moreover, ectopic expression of Oct-4 in heterologous cell system has been shown to transform nontumorigenic cells and induces tumorigenicity in nude mice, implicating its possible role in neoplastic process.⁴⁰ Recently, Qi Wang et al, 2009 have shown Oct-4 protein expression in 17.9% of ESCC patients to be associated with higher histological grade and poorer clinical survival.⁴¹ The elevated expression of Oct-4 observed in 92% of ESCCs and 62.5% of preneoplastic esophageal lesions in the present study emphasizes its role in early stages of esophageal tumorigenesis. Interestingly, 12/17 (70%) of the ESCC sera samples showed expression of Oct-4 mRNA as compared to no detectable expression in sera of normal subjects. The presence of this stem cell marker in the sera of the patients showing evidence of dysplasia and hyperplasia implicates its possible potential as a marker for noninvasive early

diagnosis of this disease. Earlier, Oct-4 overexpression has been shown to be positively associated with the recurrence in patients with preoperative CRT in rectal carcinoma by Saigusa et al 2009.⁴² Thus, further analysis of Oct-4 expression in sera of patients who have been given CRT can be carried out to analyze its potential as a noninvasive marker for monitoring relapse and response to therapy.

Sox-2, the master of pluripotency control has recently been identified as a novel major oncogene recurrently amplified and activated in the squamous cell carcinoma. These studies have used a similar strategy of chromosomal aberrations screening to identify the Sox-2 locus alone of the most frequently amplified site over the SCC genome. It has been further highlighted by Hussenet et al 2010 that the recurrent Sox-2 activation and its necessary role for squamous cell carcinoma survival.⁴³ Finally, Yun Lu et al 2009 showed that Sox-2 is also involved in the early steps of lung SCC, as participating to transform human bronchialepithelial cells.⁴⁴ Furthermore, Sox-2 overexpression can induce the expression of the squamous markers p63 and keratin 6, supporting the idea that Sox2 might be implicated in SCC differentiation.⁴⁵ Sox-2 has been found to be an immunogenic antigen in 41% of small cell lung cancer patients⁴⁶ and in 29% of meningioma patients.⁴⁷ Immunohistochemistry results suggest that Sox-2 is involved in later events of carcinogenesis, such as invasion and metastasis of pancreatic intraepithelial neoplasia.⁴⁸ Sox-2 may also be involved in gastric carcinogenesis⁴⁹ and may be amplified in prostate cancers.⁵⁰ Furthermore, Sox-2 expression has been observed in 43% of basal cell-like breast carcinomas and was found to be strongly correlated with CK5/6, EGFR, and vimentin immunoreactivity, suggesting that Sox-2 may play a role in conferring a less differentiated phenotype in these tumors.¹¹ The genomic peak amplification of the 3q26.33 found in SCC is also found in ESCC and contains the transcription factor Sox-2 which is mutated and responsible for the hereditary malformations. In the present study the elevated expression of Sox-2 was found in 95% cases and moreover, the expression of Sox-2 is mainly in the cancerous tissues it is either very low or absent in distant normals. Our results are in accordance with the latest study of Qi Wang et al 2009 in which 22.84% patients showed elevated results.⁴¹ The study



can be further carried out in order to see the stable expression of Sox-2 in the radioresistant and chemoresistant cells. This will corroborate the oncogenic potential further.

Nanog another transcription factor which plays an important role in maintain the pluripotency of the embryonic stem cells and it has been found that it has strong correlation with Sox-2 and Oct-4 in its expression. The expression of Nanog has been studied in many cancers including the colorectal cancer in which it shows higher expression in a significant number of patients and is associated with poor prognosis, lymph node metastasis and invasion. Nanog has also been associated with OSCC.⁵¹ To the best of our knowledge, present study is the first report showing the increased expression of Nanog in esophageal squamous cell carcinomas 67%. Elevated levels of circulating Nanog mRNA in the sera of the cancer patients observed for the first time in the present study may also implicate its potential as noninvasive biomarker with 10/17 (60%) of the cases showing expression of this transcript which is entirely absent from the normal sera. However, the results need to be corroborated in larger cohort of patients.

Bmi1 mRNA expression was 75% in the ESCC samples. The level of Bmi1 mRNA expression was found to be elevated in all the dysplastic and hyperplastic tissue samples analyzed along with true carcinomas. This indicated that Bmi1 plays an important role in the initial stages of development of ESCC, and may be of diagnostic value. However, its expression needs to be validated in a larger cohort for better understanding of clinical significance of its expression in ESCCs. The Bmi1 mRNA expression was also found to be elevated in some sera samples of ESCC patients as compared to normal sera, the percentage of sera samples with elevated expression was less than 50%, and this could be due to the low presence of circulating Bmi1 mRNA in ESCC patients or due to the low levels of total circulating mRNAs in sera.

Conclusion

In conclusion, overexpression of stemness markers in preneoplastic esophageal tissues and ESCCs as compared to non-malignant esophageal epithelium suggests that they may serve as a candidate

molecular target for early detection of esophageal cancer. Esophageal cancer patients often report to the clinics in advanced stages of the disease (often inoperable due to extensive metastasis); hence availability of clinical human specimens in early stages of the disease (dysplasia and hyperplasia) is severely limited. In view of these constraints the present study is of great significance. However, in-depth studies are warranted to determine their potential as candidate molecular markers for esophageal cancer. Furthermore, the detection of elevated levels of circulating mRNAs of Oct-4 (70%) and Nanog (60%) in sera of esophageal cancer patients indicates their potential as minimally invasive diagnostic markers for esophageal cancer. Hence, the present study undertaken may open new avenues for in depth analysis and validation of the stem cell related genes, studied herein, as potential diagnostic tools for the identification of esophageal squamous cell carcinoma at early stages to improve its prognosis.

Note

**represents significant value.

Author Contributions

Conceived and designed the experiments: RS. Analyzed the data: KB, RS. Wrote the first draft of the manuscript: KB. Contributed to the writing of the manuscript: KB, RS. Agree with manuscript results and conclusions: KB, RS, AS. Jointly developed the structure and arguments for the paper: KB, RS. Made critical revisions and approved final version: RS. All authors reviewed and approved of the final manuscript.

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and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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