

RESEARCH ARTICLE

Comparative Analysis of Oct4 in Different Histological Subtypes of Esophageal Squamous Cell Carcinomas in Different Clinical Conditions

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Abstract

Background: Esophageal squamous cell carcinoma (ESCC) is a common cancer with poor prognosis. It has been hypothesized that Oct4 positive radioresistant stem cells may be responsible for tumor recurrence. Hence, we evaluated Oct4 expression in ESCC in pre-treatment, post neo-adjuvant residual and post-surgical recurrent tumours. **Materials and Methods:** Endoscopic mucosal biopsies were used to study Oct4 expression and the observations were correlated with histological tumor grades, patient data and clinical background. **Results:** All patients presented with dysphagia with male predominance and a wide age range. Majority of the patients had intake of mixed diet, history of alcohol and tobacco intake was documented in less than half of the patients. Oct 4 expression was significantly higher in poorly differentiated (PDSCC) and basaloid (BSCC) subtypes than the other better differentiated tumor morphology. Oct4 was also expressed by adjoining esophageal mucosa showing low grade dysplasia and basal cell hyperplasia (BCH). Biopsies in PDSCC and BSCC groups were more likely to show a positive band for Oct4 by polymerase chain reaction (PCR). Dysplasia and BCH mucosa also showed Oct4 positivity by PCR. All mucosal biopsies with normal morphology were negative for Oct4. Number of tissue samples showing Oct4 positivity by PCR was higher than that by the conventional immunohistochemistry ($p>0.05$). Oct4 expression pattern correlated only with tumor grading, not with other parameters including the clinical background or patient data. **Conclusions:** Our observations highlighted a possible role of Oct4 in identifying putative cancer stem cells in ESCC pathobiology and response to treatment. The implications are either *in vivo* existence of Oct4 positive putative cancer stem cells in ESCC or acquisition of cancer stem cell properties by tumor cells as a response to treatment given, resulting ultimately an uncontrolled cell proliferation and treatment failure.

Keywords: Esophageal squamous cell carcinoma - Oct4 - cancer stem cell

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the eighth most common cancer worldwide and the fifth most common cancer in developing countries (Parkin et al., 2005). ESCC shows great variation in geographic distributions with a remarkably higher incidence in South East Asian and Latin American countries, and eastern Himalayas (Li et al., 1980; Parkin et al., 2001). These wide geographical variations reflect a strong environmental influence. In developing countries, ESCC is associated with poor outcome due to advanced stage disease, high recurrence and also resistance to treatment (ICMR., 2002; Enzinger et al., 2003). Literature cites that only a small fraction of the tumor is associated with tumor re-growth and these cells are believed to be clonal origin which undergoes self renewal and differentiation, as in normal stem cells (Ghisolfi et al., 2012; Wicha, 2014). Zhang et al.

(2008) observed that radio resistant esophageal carcinoma cell lines are positive for Oct4. ESCC tumor cells are heterogeneous population on histology mimicking the multi potent characters of cancer stem cells (CSC) (Gao et al., 2008). Oct4 is a member of POU-domain transcription factors and is expressed normally by pluripotent cells of embryonic tissue (Nichols et al., 1998) and adult stem cells (Niwa et al., 2000; Warthemann et al., 20012). Oct4 has important role in maintenance and controlling of embryonic stem cell pluripotency including childhood germ cell tumors (Jones et al., 2004) and a few adult carcinomas (Atlasi et al., 2006; Cheng et al., 2007; 2009). Hu et al. (2008) successfully inhibited tumor growth by decreasing cancer like stem cell population with the help of small interfering RNAs to knock down the Oct4 gene.

Under normal physiological conditions, all maturing and matured cells loses the property of Oct4 expression. Recently, it has been observed in cell culture study that a

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mature epithelial cell acquiring or expressing Oct4, is likely to get transformed to a dysplastic cell as it fails to undergo differentiation (Hochedlinger et al., 2005; Koukourakis et al., 2012), thereby suggesting the critical role of Oct4 in tumorigenesis. Recent studies have shown existence of subpopulations of cells having self renewal capacity and are believed to be the purported cancer stem cells (CSCs) (Monk and Holding, 2001; Xi et al., 2011). The CSCs are believed to undergo uncontrolled proliferation. And hence, these cells are regarded as the cell of origin for tumor and also responsible for tumor relapse, metastasis and resistance to chemotherapeutic drugs (Sanada et al., 2006). Lee et al. (2006) also documented that chemotherapy resistance CSCs were the factor responsible for recurrence in hepatocellular carcinomas. In spite, the advancement in ESCC management, overall survival rate has not improved over the years. In many of these patients, treatment failure usually relates to relapse, recurrent or metastasis (Campbell and Villaflor, 2010). Hence, it is the need of the hour looking for a good alternative therapeutic modality that can curtail tumor progression. More recently in 2009, CSC had been identified in the esophageal carcinoma cell line culture medium (Huang et al., 2009). Still so far, there are a few published data dealing with the significant role of Oct4 in identifying the presence of the cancer stem cell like cells in ESCC in esophageal squamous cancer cell lines (Wang et al, 2009; He et al., 2012) and very little is yet known about the potential role of Oct4 in human ESCC. A recent study had documented expression of Oct4 by cancer and hyperplastic mucosa in esophagus (Bahl et al., 2012). We believe that identification of CSC by the property of Oct4 expression in ESCC may help in better understanding the biology of the disease process. Hence, we studied expression of Oct4 in endoscopic biopsies of untreated, residual and recurrent ESCC tumor tissue, in an attempt to understand its potential role in disease pathobiology and response to treatment.

Materials and Methods

The study was approved by the Institute Ethics Committee and informed consents were obtained from all patients who were enrolled in the study in a pre-made proforma explaining the study methodology and the perspective of the plausible clinical implication. For the study purpose, endoscopic mucosal biopsies obtained during upper gastrointestinal endoscopic examination were used. We enrolled 100 patients of ESCC with the following details-I) 100 patients included were those who had pre-treatment endoscopic biopsies at the time of first upper gastrointestinal (GI) endoscopy. All 100 enrolled patients, the biopsies were histologically confirmed to be squamous cell carcinoma, II) 50 of these patients had residual disease in post neo-adjuvant therapy in endoscopic biopsy done during the process of interval re-assessment of the patients, and III) the other 50 patients were the ones who were found to have recurrent disease on post surgery follow-up endoscopy. Biopsies from the adjoining mucosa were also taken at the time of first endoscopic procedure. All patients included in the study were T2N1M0 stage.

All patients underwent platinum based neo-adjuvant chemoradiotherapy over 4 weeks time. Following which all underwent restaging work-up including upper GI endoscopic examination. Any suspicious mucosal lesion or residual tumor observed was biopsied for histopathological examinations. Patients who were found fit for surgery, were subjected to esophagectomy within 4 to 8 week's time. Post operatively, all patients were followed up at every two months with chest X-ray, if required with computerized tomographic examination and upper GI endoscopy. Re-biopsy was done in any patient with a suspicious focus during followed-up upper GI endoscopic examination. The present study included the patients who had recurrence of the disease within 12 months time after the surgery.

All biopsies were subjected to serial frozen sectioning and the first section in every case was stained with H&E for baseline assessment biopsy. Rest of the sections were fixed in 10% acetone tissue grade (Sigma Aldrich, USA) and stained with Oct4 primary antibody (Rabbit polyclonal, dilution 1:50; Abcam, Cambridge, UK) by standard peroxidase anti-peroxidase technique. EnVision™ systems (Dako, Glostrup, Denmark) was used as the detection system. Negative control sections were put up side by side without the detection system. About 50mg of fresh tissue was immediately immersed into RNA later™ solution (Sigma Aldrich, USA) and was used for RNA extraction by Qiagen's RNA extraction kit (Hilden, Germany). Quality of RNA extract was evaluated by gel electrophoresis and RNA concentration was assessed by spectrophotometer at 260nm under UV light. PCR products were separated on 1% agarose gel, stained with ethidium bromide and visualized under UV light. Primer sequences used were: sense 5'-AAGGATGTGGTCCGAGTGT-3' and anti-sense 5'-CAAAAACCCTGGCACAAACT-3' with an annealing temperature of 60°C and giving a product size of 357bp. β actin used as positive control and the primer sequence was 5'-CACGAAACTACCTTCAACTCC-3' for downstream and 5'-CATACTCCTGCTTGCTGATC-3' for upstream and the annealing temperature was 56°C with the product size of 357bp. Interpretation: Immunohistochemistry stained sections were interpreted in percentages per 1000 tumor cells counted and were graded into three groups depending on the number of positively stained tumor cells for Oct4 i.e. group I \leq 10%, group II \leq 50% and group III $>$ 50%. In non-neoplastic non-carcinomatous mucosa, positive patterns were expressed as basal cell layer, lower 1/3rd, lower 2/3rd or full thickness of the squamous epithelium. Number of biopsies showing positivity was expressed in percentages. A positive band in gel plate was interpreted as positive by PCR. The observations were compared using Chi-square test. A p-value less than 0.05 was interpreted as for significant.

Results

All patients presented for the first time with history of variable grades of dysphagia. Sex ratio (M:F) was 3:2 and age ranged from 28 to 80 years with the mean of 50 \pm 13 years and median of 52 years. Dietary habit was

mainly mixed diet comprising of both non-vegetarian and vegetarian in 60% and 40% were pure vegetarian. There was history of smoking and tobacco chewing in 30 males and 8 females, there was also history of regular alcohol consumption in 24 male patients. Family history of cancer was present in 2 patients and the tumors were located in colon and breast. Histology of squamous cell carcinomas in the 100 pre-treatment biopsies were well differentiated (WDSCC) in 45, moderately differentiated (MDSCC) in 16, poorly differentiated (PDSCC) in 21 and basaloid (BSCC) variant in 18 patients. Biopsies in 50 residual tumors were 6(12%) WDSCC, 9(18%) MDSCC, 15(30%) PDSCC and 20(40%) BSCC. Biopsies in 50 recurrent tumors were 8(16%) MDSCC, 19(38%) PDSCC and 23(46%) BSCC (Table 1). Biopsies from adjoining mucosa showed normal morphology in 65, basal cell hyperplasia (BCH) in 32 and low grade dysplasia in 3. Site wise distributions of 100 tumors were middle 3rd in 54, lower 3rd in 34 and upper 3rd in 12 patients.

Tissue distribution of Oct4 protein: oct4 exhibited nuclear positivity. Oct4 was negative in all the biopsies showing normal morphology. Amongst the biopsies with BCH, 14 were negative and 18 showed nuclear positivity confined to proliferative basal cells (Figures 1A and 1B). Ten of the 32 biopsies (31%) with BCH also showed positive band in PCR and all these 10 biopsies showed nuclear Oct4 positive staining in immunohistochemistry. The three biopsies with low grade dysplasia were positive for Oct4. Oct4 positivity in different benign mucosa was significantly different. PCR was positive in all 3 dysplasia and 17/57(30%) BCH. All normal mucosa were negative. (Table 2, Chi Square test, $p < 0.0001$) Carcinoma cells showed variable degree of staining intensity suggesting heterogeneity of the tumor cells. Distributions of the Oct4 in different subtypes of SCC are shown in Table 3. BSCCs expressed stronger intensity and 80 to 99% tumor cells showed positive staining. Percentage of Oct4 positive tumor cells were lesser in well differentiated tumors and the differences in Oct4 positive cells were significantly different amongst different grades tumors, higher percentage of positive cells were observed in less differentiated tumors including BSCC (Chi square, $p < 0.0001$) Recurrent and residual tumors showed higher percentages of Oct4 positive cells. PCR for Oct4 gene was positive in 89% of untreated tumor tissue and 100% of recurrent and residual tumors. Amongst the positive cases, intensity and size of the bands were more prominent in carcinoma tissue than non carcinoma. Amongst the clinical parameters, recurrent and residual tumors showed significantly higher percentages of Oct4 positivity;

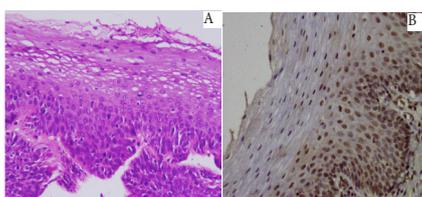


Figure 1. Photomicrograph Showing. A) Basal Cell Hyperplasia in a Biopsy Taken from the Adjoining Mucosa, (H&E, x450); B) Nuclear Positivity for Oct4 Confined to Lower Half of the Proliferative Zone (PAP, x450)

Table 1. Break-up of the Histological Types of the Carcinoma in Different Clinical Groups

Tumor type	Pre-treatment biopsy	Residual tumor	Recurrent tumor	Total No. (%)
WDSCC	45	6	0	51 (25.5)
MDSCC	16	9	8	33 (16.5)
PDSCC	21	15	19	55 (27.5)
BSCC	18	20	23	61 (30.5)
Total	100	50	50	200 (100)

*Chi Square test=57.54; $p < 0.0001$; WDSCC=well differentiated squamous cell carcinoma; MDSCC=moderately differentiated squamous cell carcinoma; PDSCC=poorly differentiated squamous cell carcinoma; BSCC=basaloid squamous cell

Table 2. Histological Features of Mucosal Biopsies from Adjoining Areas and Distribution of Oct4 Positive Cases in Immunohistochemistry Staining

Histological features (number of biopsy)	Oct4 immunohistochemistry		Oct4 PCR	
	ve+	ve-	ve+	ve-
Normal mucosa (65)	0	65	0	65
Basal cell hyperplasia (32)	18	14	10	22
Low grade dysplasia (3)	3	0	3	0
Total (100)	21	79	13	87
Chi Square test/p value	68.5	<0.0001	27.74	<0.0001

Table 3. Distributions of Oct4 Positivity Amongst Different Subtypes of Carcinomas

Carcinoma subtype (No.)	Oct4+ive by Immunohistochemistry staining			Oct4+ive by PCR (%)	
	Negative	Group		I	II
		I	II		
WDSCC (51)	28	23	0	0	14/20 (70)
MDSCC (33)	2	18	13	0	28/31 (90.3)
PDSCC (55)	0	5	28	22	30/35 (85.7)
BSCC (61)	0	9	14	43	45/45 (100)
Total = 200	30	65	55	65	117/131 (89.3)

*Chi Square test=57.54; $p < 0.0001$; **WDSCC=well differentiated squamous cell carcinoma; MDSCC = moderately differentiated squamous cell carcinoma; PDSCC =poorly differentiated squamous cell carcinoma; BSCC=basaloid squamous cell carcinoma; Tx bx=treatment biopsy; tm=tumor; IHC=immunohistochemistry; PCR=polymerase chain reaction

Table 4. Distribution of Oct4 by Immunohistochemistry and PCR in Different Groups

Tumor type	Pre-tx bx	Residual tm	Recurrent tm	Total No. (%)	Oct4+ive by	
					IHC (%)	PCR (%)
WDSCC	45	6	0	51 (25.5)	23	18
MDSCC	16	9	8	33 (16.5)	31	55
PDSCC	21	15	19	55 (27.5)	55	55
BSCC	18	20	23	61 (30.5)	61	61
Total	100	50	50	200 (100)	170 (85)	189 (94.5)

*Chi Square test=57.54; $p < 0.0001$; **WDSCC=well differentiated squamous cell carcinoma; MDSCC = moderately differentiated squamous cell carcinoma; PDSCC =poorly differentiated squamous cell carcinoma; BSCC=basaloid squamous cell carcinoma; Tx bx=treatment biopsy; tm=tumor; IHC=immunohistochemistry; PCR=polymerase chain reaction

other clinical variables including the time gap between completion of CRT and the day of operation, detection of the tumor recurrence and the pathological features, did not show any significant influence in oct4 positive status.

Discussion

The emerging concept and understanding the role of cancer stem cell (CSC) in tumor development and progression may influence the diagnostic and therapeutic approaches in esophageal cancer management. Role of CSCs in tumorigenesis is believed to be generated through uncontrolled self renewal of normal stem cell population or the progenitor cell (Gao et al., 2008; Zhang et al., 2008;

Ghisolfi et al., 2012; Wicha, 2014). In the present study, we examined the patterns of Oct4 expressions in biopsies taken from human ESCC under different clinical settings and also esophageal mucosa taken adjacent to the primary tumor focus by immunohistochemistry and conventional PCR techniques. We observed significantly higher number of Oct4 positive cells in BSCC and PDSCC subtypes indicating higher CSC like cell populations. These observations were irrespective of the clinical background of treatment profiles. Conversion in the histological subtype of the tumor before and after neo-adjuvant chemoradiotherapy associated with higher percentages of Oct4 positive CSC like carcinoma cells in the residual and recurrent tumors, these conversion and differences in the Oct4 positive cells may be result of re-activation or uncontrolled proliferation of the small number of existing stem cell population within the tumor and these cells are known to acquire self-renewal property. These characters have been described as the inherent property of such cell, thereby acquiring oct4 expression in the process. It may also be interpreted that poorly differentiated ESCC exhibiting basaloid character possibly acquires stem cell property identified by Oct4 expression. Similar to the our observation, other groups also documented higher number of basaloid or poorly differentiated carcinoma cells expressing Oct4 in patients who either had residual or recurrent ESCC (Atlasi et al., 2006; Hu et al., 2008; Chen et al., 2009; Koukourakis et al., 2012). Koukourakis et al. (2012) and Islam et al. (2014) observed high percentages of Oct4 in recurrent oral, head and neck SCC. Similar observation was made by Zhang et al. (2008), in radio-resistant esophageal cancer cell line7 and this observation was further supported by Xi et al. (2011) by demonstrating successfully high number of Oct4 positive cells in the subculture tumorspheres of human ESCC. Similar observations were made in breast and lung cancers as well in other studies (Rosenthal et al., 2012; Huang et al., 2013). Further, in a study by Kim et al. (2013) expressions for stem cell markers including Oct4 were inducible by treatment with H₂O₂ of the human malignant mesothelial cell line. In a study by Zhou et al. (2013) using human biopsy materials of gastric origin, the Hippo signalling pathway marker mRNAs including that of Oct4 exhibited an increasing tendency from cancerous tissue to normal gastric mucosa. And the mRNA expressions had a close correlation with lymphatic metastasis and tumor TNM staging. Cancer cells are known to have properties similar to embryonic stem cells in their ability to grow indefinitely, immortality, self renewable character and invasive nature. In the present study, expression of Oct4 by recurrent and residual tumors could as well explain the radio resistant property of these tumor cells, thereby affecting the patient outcome and ultimately patient survival (Campbell and Villafior, 2010). Huang et al. (2009) proposed that Oct4 might play a role in conferring a less differentiated phenotype or inactivating the ability to differentiate by the tumor cells in esophageal cancers. They also observation Oct4 expressions by poorly differentiated carcinoma derived human esophageal squamous cancer cell line and cancer tissue, which were similar to our observation of higher Oct4 expression by higher histological grade

and poor tumor outcome. This group also observed uneven distribution of Oct4 positive cells within tumor, ranging from few scattered individual cells to cells in large aggregates. There are two studies (Tai et al., 2005; He et al., 2012) who had reported Oct4 expression by dysplastic and hyperplastic esophageal mucosa confining to the proliferative zone, and not expressed by the normal mucosa. Oct4 positivity is reported in certain adult tissue including benign endometrial stromal cells (Atlasi et al., 2006; Cauffman et al., 2006; Mathai et al., 2006; Katona et al., 2007; Zangrossi et al., 2007; Atlasi et al., 2008; Forte et al., 2009). We observed slightly higher sensitivity by PCR technique than by the conventional immunohistochemistry method in detecting Oct4 expression, which is similar to other reported data (Atlasi et al., 2006; Xi et al., 2011; Li et al., 2012). This disparity could be due to the sensitivity of the technique used as PCR is observed to be more sensitive than immunohistochemistry methods and also Oct4 is known to have two isoforms (Lee et al., 2006). Oct4 over expression had also been documented by the side population cells isolated from Lewis lung carcinoma cell line, compared to non-side population cells (Zhang et al., 2012). With the advent of new technology involving the induction of pluripotent stem cell (iPSCs), has opened up the possibility of generating patient specific stem cell lines. In this methodology, a cell can be directed to differentiate into specific type for further pathological investigation with the option of therapeutic application (Hochedlinger and Plath, 2010; Warren et al., 2010; Zhou et al., 2010). Subsequent to these reports, human cancer cell lines have been reprogrammed into iPSCs using various types of transcription factors including Oct4 for a canonical reprogramming, by introducing plasmids into mouse Lewis lung carcinoma cell lines. The cultures exhibited clusters of green fluorescent forming colonies expressing embryonic stem cell markers like Oct4 (Ilet et al., 2010). These studies indicate the feasibility of developing iPSC-based experimental cancer vaccines for immunotherapy.

In conclusion, we report differential Oct4 expression by different histological subtypes of ESCC taken from different clinical materials relating to neo-adjuvant chemoradiotherapy and adjoining non-carcinomatous esophageal mucosa. Oct4 expression was exhibited not only by the poorly differentiated tumors but also by hyperplastic and low grade dysplastic mucosa of the adjacent mucosa. Oct4 positive cancer stem cells possibly play important role in treatment response. Our study reiterates the important role attributed by Oct4 in controlling cell differentiation and proliferation by the cancer stem cell like ESCC cells.

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